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ERRATA

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Page 296:

Lines 2–9 should be inserted on page 295 between lines 1 and 2.

Page 415:

Line 18: For "not" read "now".

BIOCHIMICA ET BIOPHYSICA ACTA, VOL. 41

Page 247:

Legends of Figs. 2 and 3: For "cytogrome" read "cytochrome".

ZONE ELECTROPHORESIS IN SILICA GEL AND SILICA PASTE*

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SUMMARY

Silica gel and silica paste have been used as anticonvection media in zone electrophoresis. Rapid methods for preparing gels and pastes containing the appropriate electrophoretic buffer, and their use in zone electrophoresis, are given.

INTRODUCTION

In 1946 CONSDEN, GORDON AND MARTIN[1] used freshly prepared silica gel as an anticonvection medium for the electrophoretic separation of various amino acid and peptide mixtures. A number of separations were effected, and material in the bands was readily recovered in good yield by drying and extracting the gel with water. CONSDEN AND GORDON[2] employed silica gel in the electrophoretic separation of peptides and amino acids from hydrolysates of wool. That silica gel has not been more widely used as an anticonvection medium in electrophoresis is probably due to the length of time required for the preparation of these gels, and their fragile nature. This paper reports a simple and rapid method for the preparation of silica gels containing appropriate electrophoretic buffers and their use in electrophoresis. For certain situations in which gels cannot be employed, the preparation and use of silica pastes as an anticonvection agent is given.

PROCEDURE

Electrophoresis apparatus

A modification of the apparatus described by CRESTFIELD AND ALLEN[3] is used. Plastic side and end strips coated with silicone stopcock grease on the surfaces of contact are employed to form electrophoretic troughs on the level glass plate of the apparatus 38 cm in length and 0.6 cm in thickness. The width varies from 5 to 15 cm, depending upon the particular application.

Preparation of silica sol

Silica sol is prepared by a modification of the procedure of PRAMER[4]. A solution that is 1 M in sodium metasilicate (6 % in SiO_2) and 10^{-3} M in sodium chloride is passed through a column of amberlite IR-120 in the hydrogen form. The silicic acid sol that emerges from the column has a pH of approximately 3, and will remain as

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[*] Paper of the Journal Series, New Jersey Agricultural Experiment Station.

a sol in the refrigerator for at least two weeks. The sodium chloride is converted to hydrochloric acid, and the resultant low pH stabilizes the sol. At pH values near neutrality gelling occurs in 1 h or less at room temperature and in about one day at 4°.

Preparation of gels containing electrophoretic buffer

Electrophoretic buffers with a final ionic strength of 0.05 to 0.1 are commonly employed. One volume of silica sol is mixed with one volume of buffer that is twice the desired final concentration, and the solution is poured into the trough of the electrophoresis apparatus and allowed to gel. Gels from 2 to 6 mm in thickness have been used. The time required for gelling will depend upon the pH and ionic strength of the buffer and the temperature. A comprehensive investigation of the effects of pH, ionic strength, and temperature on the rate of gelling of silica sols has been made by PRAMER[4] whose work should be consulted for information on gelling time under various conditions. In the pH range from 5.0 to 8.5, and with an ionic strength of 0.05 to 0.1, the length of time required for the solution to gel varies froma few minutes to about 0.5 h. Gelling can be speeded by heating the solution.

Introduction of sample and electrophoresis

A slot is made in the gel for the introduction of the sample. Slots can be prepared by cutting out the gel or by incorporating a removable strip of plastic of the desired dimensions in the trough prior to casting the gel. Since the gel is rather fragile, and tends to stick to glass and plastic materials, care must be taken not to crack the gel during this operation. A method which has been found to be superior to either of the above methods is to use a thin-walled capillary tube attached to a vacuum line to aspirate material from the region where the inlay is to be made. Slots of 2 mm in width are easily made using this technique. Slots 3 to 10 mm in width and extending to within 1 cm of the sides of the slab have been used routinely. For a number of separate samples, small rectangular or circular openings can be made in the gel with the suction tube.

The pH of a liquid sample is adjusted to that of the electrophoretic buffer, and the sample is added to an equal volume of the sol. The solution is then poured into the slot, where it gels. In order to minimize syneresis and shrinking or swelling of the gel in the region of the inlay during electrophoresis, the ionic strength of the inlay should not vary markedly from that of the main body of the gel. A small amount of concentrated buffer can be added to dilute samples prior to mixing with the sol, and with samples of high ionic strength a more concentrated buffer can be employed in the main body of the gel. Solid samples, especially if they are of limited solubility, can be dissolved in sol that has been diluted with water or buffer.

Electrophoresis

The plastic strips that form the ends of the trough are removed after gel formation and filter paper pads that extend into the buffer vessels are laid on the ends of the gel. The pads are covered with a single thickness of polyethylene film up to the point where the pad contacts the gel in order to minimize evaporation from the paper. A cover plate that rests on the side strips and remains a few millimeters above the gel is placed on the apparatus, and electrophoresis is commenced. The field strength that has been employed is in the range of 10 to 20 V/cm. Generally, it is advisable to begin the electrophoretic run at lower voltages, as heating, syneresis, swelling and shrinking of the gel in the region of the inlay are aggravated by higher voltages.

Electro-osmosis is slightly less in silica gel than in paper at pH values up to 8. Above pH 8, electro-osmosis in the gel increases rapidly, until at pH 10 it is about three times that observed in Whatman No. 1 paper. Caffeine is used as an indicator of electro-osmosis and is detected by examining the slab with a short-wave u.v. light in a darkened room. After the run is completed, substances may be located within the gel by various methods. Those that absorb u.v. light can be detected by examination of the gel with an appropriately shielded u.v. light. The gel can be sprayed or stained, or a piece of filter paper that has previously been moistened with water and partially dried can be placed in contact with the surface of the gel for a short period of time and the bands printed off onto the paper. The paper is then dried and the location of the bands determined by the appropriate technique.

The use of silica at pH values some distance from neutrality

Silicic acid sols gel at extremely slow rates at pH values less than 5 or greater than 8. Electrophoresis is carried out in silica paste in these regions. A suitable paste can be prepared by either of the following procedures.

1. An amount of sol, three times the quantity that would be used in preparing the slab of gel, is gelled in a beaker by the addition of a component of the electrophoretic buffer that is approximately neutral in pH (for example: sodium monochloroacetate, in the case of monochloroacetic acid–sodium monochloroacetate buffer of pH 2.3). The gel is stirred with a motor-driven stirrer until smooth and the remaining component of the buffer is added to give the desired final pH and ionic strength. The silica gel, now in the form of a thin paste, is centrifuged (2300 × *g*, 5 min) and the supernatant liquid is decanted. The thickened paste, which contains three times the SiO_2 content of the original gel, is stirred with a spatula to break up lumps and is spread in the electrophoretic trough.

2. CAB-O-SIL M-5 (Godfrey L. Cabot, Inc., Boston, Mass.), a colloidal form of silica, is stirred with complete electrophoretic buffer until a stiff paste is formed. Approx. 12 g of CAB-O-SIL are required /100 ml of buffer. This paste is then spread in the trough.

A slot is formed in the paste for introduction of the sample. The sample, in solution, is mixed with an equal volume of paste and poured into the slot. The paste in the inlay can be thickened by the addition of a small amount of CAB-O-SIL. If a wide slot is used, the sample can be made up as a thick paste by the addition of CAB-O-SIL prior to addition to the slot.

Removal of substances from the gel or paste

Nucleotides and amino acids, the substances which have been investigated in this work, are easily removed from the silica by any of three methods. The gel may be dried in air or in an oven and the nucleotide or amino acid extracted with a small amount of water. The gel and paste can be lyophilized and the material extracted, a procedure that should be useful when working with labile substances. Material can be recovered in a more dilute form by homogenizing the undried gel or paste with a few volumes of water and centrifuging the solid residue.

Comparison of gel and paste

The silica gel is more transparant than the paste, is free of air-bubbles and is more uniform in texture. It is easily cracked and cannot be repaired if broken. The smoothness and clarity of the gel permit bands to be easily detected by scanning or other techniques. The paste is less brittle than the gel and if cracking or other accidents occur, they can be repaired by working the paste with a spatula.

For large scale separations with silica paste, it is advantageous to place the top lid in contact with the paste. The trough is completely filled with paste and a glass cover plate is tilted from the side onto the surface of the paste. The plate is removed after the run is completed by sliding it sideways off the paste.

RESULTS AND DISCUSSION

Fig. 1. (left) shows a large-scale separation in silica paste of the four major ribonucleotides. The bands were printed off onto filter paper which was then photographed using a short-wave u.v. light. Paste in the region of the bands was removed from the slab, dried in an oven at 55°, and the nucleotides extracted with water. Recoveries were as follows: cyticylic acid 81 %, adenylic acid 93 %, guanylic acid 94 %, and uridylic acid 96 %. The rather low recovery of cytidylic acid is probably due to losses in the region of the soft inlay.

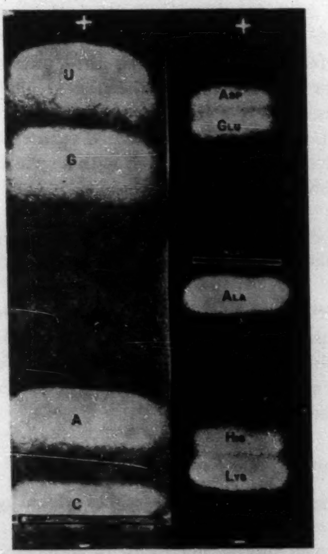

Fig. 1. Separation of ribonucleotides and amino acids by electrophoresis in silica paste and gel. Left: 20 mg each of uridylic acid (U), guanylic acid (G), adenylic acid (A) and cytidylic acid (C). Conditions: Silica paste as the anti-convection medium; 0.05 *M* formate buffer, pH 3.4; 38 × 10 × 0.6 cm slab; 12 V/cm for 4 h; glass plate in contact with the upper surface. Right: Separation of 5 mg each of aspartic acid, glutamic acid, histidine and lysine, and 7.5 mg of alanine in silica gel. Conditions: 0.1 *M* acetate buffer, pH 5.5; 38 × 8.5 × 0.2 cm slab; 12 V/cm for 2 h.

Fig. 1. (right) shows a separation in silica gel of a mixture of five amino acids. The bands were printed off onto filter paper and visualized with ninhydrin. In a second experiment, 5-mg quantities of aspartic acid, alanine and lysine were separated under similar conditions. A print was made and the bands were removed, dried at 55°, and the amino acids extracted. Recoveries, as determined by the ninhydrin method of MOORE AND STEIN[5], were as follows: aspartic acid 96 %, alanine 99 %, and lysine 95 %.

Electrophoresis in silica gel or paste would appear to be a useful method for the purification of small amounts of materials, owing to the speed of separation, the ease with which substances can be recovered from the silica, and the lack of contamination of the separated substances with material from the anticonvection medium. These procedures should also be adaptable to quantitative techniques, as the gel or paste do not retain substances of low molecular weight that are added to them.

GORDON *et al.*[6] report that proteins cannot migrate electrophoretically through the pores of silica gel, and these results have been confirmed in this laboratory with ribonuclease, cytochrome *c*, serum albumin, and hemoglobin, all of which remain at the site of application in the gel or paste. However, the ribonucleic acid from yeast that is soluble in 1 *M* sodium chloride and which has been shown to be of about 9 nucleotides in length[7] does migrate in the gel without restriction, as compared to its mobility on paper. If, as suggested by GORDON *et al.*[6], proteins are unable to migrate in silica gel due to their large size, it would appear that in the molecular weight region somewhat above that of the soluble RNA (about 3,000) electrophoretic fractionations might be achieved by molecular sieve effects in which the size and shape of a molecule as well as the charge density would dictate its rate of movement in the gel. Such sieving effects have been noted in the migration of certain proteins in starch gels of high concentration[8].

ACKNOWLEDGEMENTS

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REFERENCES

[1] R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 40 (1946) 33.
[2] R. CONSDEN AND A. H. GORDON, *Biochem. J.*, 40 (1950) 8.
[3] A. M. CRESTFIELD AND F. W. ALLEN, *Anal. Chem.*, 27 (1955) 422.
[4] D. PRAMER, *Appl. Microbiol.*, 5 (1957) 392.
[5] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
[6] A. H. GORDON, B. KEIL, K. SEBESTA, O. KNESSL AND F. ŠORM, *Coll. trav. chim. tchécoslov.*, 15 (1950) 1.
[7] F. F. DAVIS, A. F. CARLUCCI AND I. F. ROUBEIN, *J. Biol. Chem.*, 234 (1959) 1525.
[8] H. G. KUNKEL AND R. TRAUTMAN, in M. BIER, *Electrophoresis*, Academic Press, New York, 1959, p. 236.

FURTHER INVESTIGATIONS ON A $Mg^{++} + Na^{+}$-ACTIVATED ADENOSINTRIPHOSPHATASE, POSSIBLY RELATED TO THE ACTIVE, LINKED TRANSPORT OF Na^{+} AND K^{+} ACROSS THE NERVE MEMBRANE

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SUMMARY

The sodium activated ATPase prepared from crab nerve which was described in a previous paper[1] has been subjected to a more detailed study of the relation between enzyme, substrate, and a number of monovalent and divalent cations. It has also been investigated whether the enzyme catalyses an ATP-Pi or an ATP-ADP exchange reaction, and whether these reactions are cation-dependent. Finally, the effect of g-strophanthin on the ATPase activity and the exchange reaction has been investigated.

The simplest scheme by which the experimental results can be explained is as follows:

$$E + ATP + 2\,Mg + n\text{Na} + m\text{K} \rightleftharpoons \underset{n\text{Na}}{\overset{m\text{K}}{EMg_2ATP}} \qquad (1)$$

$$\underset{n\text{Na}}{\overset{m\text{K}}{EMg_2ATP}} \rightleftharpoons \underset{n\text{Na}}{\overset{m\text{K}}{E}} \sim P + 2\,Mg + ADP \qquad (2)$$

$$\underset{n\text{Na}}{\overset{m\text{K}}{E}} \sim P \rightarrow E + Pi + n\text{Na} + m\text{K} \qquad (3)$$

where n and m are unknown numbers, and where

$$\underset{n\text{Na}}{\overset{m\text{K}}{EMg_2ATP}}$$

symbolises an enzyme–substrate–ion complex in which Mg^{++} is part of the enzyme–substrate complex and in which Na^{+} and K^{+} are attached to two different sites on the enzyme; these sites show high affinities for the respective ions.

Phosphorylation of the enzyme, steps (1) + (2), will take place also when Mg^{++} is the only cation present in the system, but under these circumstances the release of inorganic phosphate, step (3), is very slow. The rate of step (3) is increased by the addition of Na^{+} to the system, but not by the addition of K^{+}. In the presence of $Mg^{++} + Na^{+}$, however, addition of K^{+} leads to a considerable increase of the rate of step (3).

The binding of $Na^{+} + K^{+}$ to the enzyme takes place during the attachment of ATP to the enzyme, *i.e.* during step (1).

G-strophanthin has no effect on the phosphorylation of the enzyme, steps (1) + (2). But g-strophanthin inhibits the activating effect of Na^+ and of $Na^+ + K^+$ on step (3), presumably by interfering with the binding of the cations to the enzyme during step (1).

The observations lend further support to the suggestion made previously that this enzyme is involved in the active, linked transport of Na^+ and K^+ across the nerve membrane.

INTRODUCTION

In previous experiments[1] it has been shown that from peripheral crab nerve can be prepared an enzyme which is activated by $Mg^{++} + Na^+$ and which in the presence of $Mg^{++} + Na^+$ is stimulated by K^+. The enzyme is an adenosintriphosphatase, it is located in submicroscopic particles, and it possesses such properties that the suggestion has been made that this enzyme is involved in the active transport of Na^+ and K^+ across the nerve membrane.

Experiments recently published by POST[2] lend support to this assumption. Also the red blood cell membrane contains an adenosintriphosphatase which is stimulated by $Na^+ + K^+$ and which has properties that make it reasonable to assume that the enzyme is involved in the active transport of Na^+ and K^+ across the cell membrane.

In the present experiments the properties of the nerve enzyme have been studied more closely, particularly with a view to its possible participation in active ion transport. The studies have been concerned with the relation between enzyme, substrate, and a number of monovalent and divalent ions. It has furthermore been investigated whether the enzyme catalyses an ATP–Pi or an ATP–ADP exchange reaction and how the exchange reaction is dependent on the cations; this was done to obtain information about the intermediate steps in the breakdown of ATP and the influence of the cations on these steps.

EXPERIMENTAL

The enzyme was prepared as previously described by homogenisation of crab nerve in 0.25 *M* sucrose and differential centrifugation. The enzyme is contained in the submicroscopic particle fraction which is obtained by centrifugation at 20,000 × *g* for 1–2 h of the supernatant after centrifugation for 15 min at 10,000 × *g*. In the present experiments the enzyme was cooled to —15° immediately after the preparation and stored at this temperature until use. By this method it is possible to preserve the activity of the enzyme for months. The enzyme was used immediately after thawing, and only once frozen enzyme was used.

The ATP and ADP solutions were prepared and the enzymic activity determined as described previously, except that the activity of the enzyme was measured at 30° instead of at 36°. The ITP solutions (sodium salt from Pabst) were prepared in the same way as the ATP solutions.

Abbreviations used: ADP and ATP, adenosine-5′-di- and tri-phosphate; IDP and ITP, inosine-5′-di- and triphosphate; Pi, inorganic orthophosphate; Versene, ethylenediaminetetraacetate; Amidol, 2,4-diaminophenol, dihydrochloride.

Apart from Cs^+, the added cations in the concentrations used did not influence the determinations of Pi under the experimental conditions. Nor did Cs^+ exert any influence when the reducing substance, Amidol, was added to the solution before molybdate. On the other hand, if Amidol was added to the solution after molybdate, high concentrations of Cs^+ in the solution led to precipitates. All cations were added as chlorides.

The enzymic activity is expressed in terms of μmoles Pi split off from ATP in 30 min.

Carrier-free ^{32}P was obtained from Philips, Amsterdam.

$AD^{32}P$ was prepared according to COOPER AND LEHNINGER[3].

The exchange reactions were determined in a total volume of 1 ml containing 0.1 ml of the enzyme solution. The reaction mixture was adjusted to pH 7.2 as in the determination of ATPase activity. The ATP concentration was 3 mmoles/l in the investigations of both the ATP–Pi and the ATP–ADP exchange reactions.

In the investigations of the ATP–Pi exchange reaction, Pi was added to the reaction mixture to final concentrations ranging from 1 to 10 mmoles/l; the Pi was labelled with ^{32}P, $5 \cdot 10^5$ counts/min. In some of the experiments ADP was further added to a final concentration of 1 mmole/l. In the investigations of the ATP–ADP exchange reaction, ADP was added to a final concentration of 1 mmole/l; the ADP was labelled with $[^{32}P]ADP$, $5 \cdot 10^5$ counts/min. In some of the experiments Pi was also added to final concentrations ranging from 1–10 mmoles/l.

The incubation time was 20 min at 30°. During this period the exchange reaction varied linearly with time. The reaction was stopped by heating the mixture to 80–85°. After cooling, 0.6 ml of the mixture was diluted to 10 ml and placed on a column of Dowex 1. The nucleotides were eluted according to COHN[4]. The radioactivity was measured on 10 ml of each eluate in a liquid counter (type M6, manufactured by 20th Century Electronics).

The amount of Pi or ADP exchanged was calculated by multiplying the average concentration of Pi or ADP during the incubation by the fraction of ^{32}P or $AD^{32}P$ incorporated into ATP. Correction was made for the small amount of breakdown of labelled ATP during the incubation; the correction was obtained by multiplying the average specific activity of ATP by the amount of ATP hydrolysed.

Adenylate kinase activity was determined by the method of KALCKAR[5].

RESULTS

ATPase activity

Monovalent cations: It has been shown previously[1] that the enzyme exhibits some ATPase activity in the presence of Mg^{++} alone, but the activity is only slight. With Mg^{++} present in the system, addition of Na^{++} results in an increase of the activity. If K^+, Rb^+, Cs^+, or NH_4^+ are added instead of Na^+ the activity shows only a very small increase, while addition of Li^+ leads to an increase in activity intermediate between activities produced by Na^+ and by the other monovalent cations (Fig. 1). Maximum activity with Na^+ is obtained at a concentration of 6 mmoles/l. The Na^+ concentration at half maximum activity is 1.4 mmoles/l, and it is independent of the MgATP concentration. The concentrations of the other monovalent cations, at half maximum activity is about 10 times as high. Na^+ seems accordingly to be the monovalent cation which gives the highest increase in activity and to which the enzyme has the highest affinity.

When the system contains both Mg^{++} and Na^{+}, addition of one of the other monovalent cations, NH_4^+, K^{+}, Rb^{+}, Cs^{+} or Li^{+}, leads to a considerable increase of the activity of the enzyme (Figs. 2 and 3). This effect is only obtained with Na^{+} in the solution. If Na^{+} is replaced, by, for example, Li^{+}, addition of one of the other cations does not lead to an increase in the activity, but to an inhibition of the activity due to Li^{+}.

The stimulation produced by K^{+}, Rb^{+}, Cs^{+}, NH_4^+ and Li^{+} depends on the Na^{+}

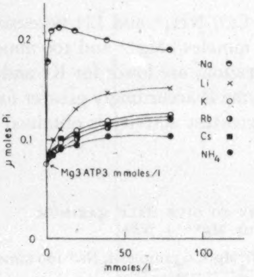


Fig. 1. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++}. In Figs. 1–3 and 5–13 the enzyme activity is expressed as µmoles inorganic phosphate split off ATP within 30 min under the experimental conditions. ATP concentration: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

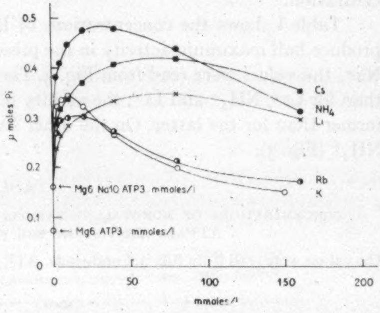


Fig. 2. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++} + Na^{+}. ATP concentration: 3 mmoles/l. Cations present: 6 mmoles/l Mg^{++} + 10 mmoles/l Na^{+}.

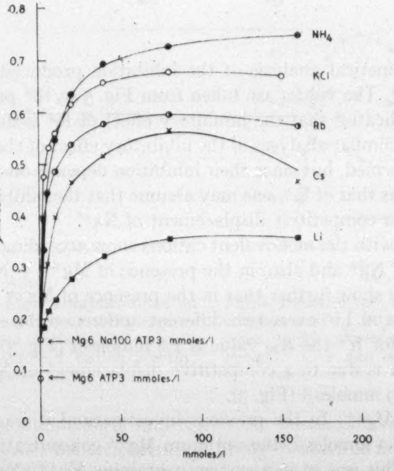


Fig. 3. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++} + Na^{+}. ATP concentration: 3 mmoles/l. Cations present: 6 mmoles/l Mg^{++} + 100 mmoles/l Na^{+}.

concentration (Figs. 2 and 3). At low concentrations of Na^+, *e.g.* 10 mmoles/l (Fig. 2), the addition of low concentrations of these cations leads to an increase of the activity, while the activity decreases again when higher concentrations are added. As has been found previously with K^+, sufficiently high concentrations of the cations lower the activity to the level observed when only Mg^{++} is present in the system.

When the Na^+ concentration is raised, the maximum activity obtainable by addition of the other monovalent cations increases until the Na^+ concentration is equal to or higher than 100 mmoles/l (Fig. 3). The concentrations of the monovalent cations which correspond to the activity maxima also increase with the Na^+ concentration.

Table I shows the concentrations of K^+, Rb^+, Cs^+, NH_4^+ and Li^+ necessary to produce half maximum activity in the presence of 6 mmoles/l Mg^{++} and 100 mmoles/l Na^+; the values were read from Fig. 3. The concentrations are lower for K^+ and Rb^+ than for Cs^+, NH_4^+ and Li^+; the affinity of the enzyme is accordingly greater for the former than for the latter. On the other hand, the greatest activity is obtained with NH_4^+ (Fig. 3).

TABLE I

CONCENTRATIONS OF MONOVALENT CATIONS NECESSARY TO GIVE HALF MAXIMUM ATPASE ACTIVITY IN THE PRESENCE OF $Mg^{++} + Na^+$

The values were read from Fig. 3. Conditions: ATP 3 mmoles/l, Mg^{++} 6 mmoles/l, Na^+ 100 mmoles/l.

Cation	*Concentration (mmoles/l)*
Li^+	15.0
NH_4^+	5.0
K^+	1.8
Rb^+	2.1
Cs^+	8.0

Fig. 4 shows a kinetical analysis of the inhibition produced by K^+ at various concentrations of Na^+. The values are taken from Fig. 5 in the previously published paper[1]. The graph indicates[6] that the inhibitory effect of K^+ is due to a competitive displacement of Na^+. Similar analyses of the inhibitory effect of the other monovalent cations were not performed, but since their inhibition depends on the Na^+ concentration in the same way as that of K^+, one may assume that the inhibitory effect of these cations is also due to a competitive displacement of Na^+.

The experiments with the monovalent cations show accordingly that the enzyme has a high affinity for Na^+ and also, in the presence of $Mg^{++} + Na^+$, a high affinity for K^+ and Rb^+. They show further that in the presence of $Mg^{++} + Na^+$ the cations K^+, Rb^+, Cs^+, NH_4^+ and Li^+ exert two different actions on the enzyme. One action is a stimulation, and for K^+ the K_m value is 1.8 mmoles/l (Fig. 3). The other action is an inhibition which is due to a competitive displacement of Na^+; the latter has for K^+ a K_I value of 9 mmoles/l (Fig. 5).

Divalent cations. Mg^{++}: In the previous investigation[1] it was found that at an ATP concentration of 3 mmoles/l, the optimum Mg^{++} concentration was also of the order of 3 mmoles/l. This was so in a system containing Mg^{++} alone or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$. When the concentration of Mg^{++} exceeded that of ATP the activity was inhibited, and the same was observed when the ATP concentration exceeded the Mg^{++} concentration. This optimum 1/1 ratio between Mg^{++} and ATP is also found at other concentrations of Mg^{++} and ATP (Fig. 5).

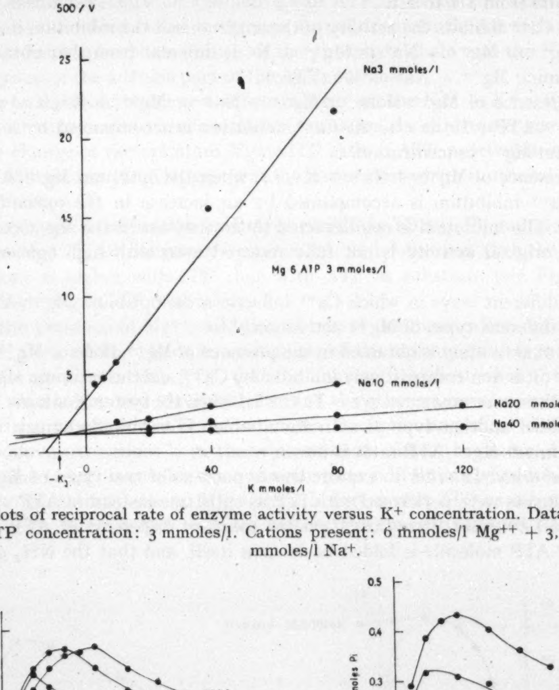
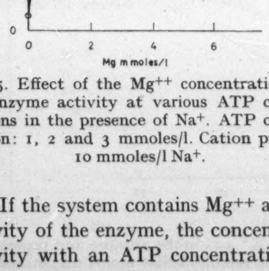


Fig. 4. Plots of reciprocal rate of enzyme activity versus K^+ concentration. Data from Fig. 5 in ref. 1. ATP concentration: 3 mmoles/l. Cations present: 6 mmoles/l Mg^{++} + 3, 10, 20 and 40 mmoles/l Na^+.



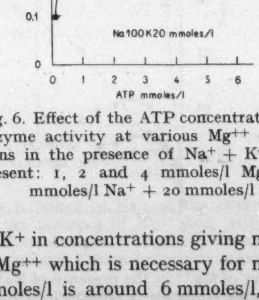


Fig. 5. Effect of the Mg^{++} concentrations on the enzyme activity at various ATP concentrations in the presence of Na^+. ATP concentration: 1, 2 and 3 mmoles/l. Cation present: 10 mmoles/l Na^+.

Fig. 6. Effect of the ATP concentration on the enzyme activity at various Mg^{++} concentrations in the presence of $Na^+ + K^+$. Cations present: 1, 2 and 4 mmoles/l Mg^{++} + 100 mmoles/l Na^+ + 20 mmoles/l K^+.

If the system contains Mg^{++} and $Na^+ + K^+$ in concentrations giving maximum activity of the enzyme, the concentration of Mg^{++} which is necessary for maximum activity with an ATP concentration of 3 mmoles/l is around 6 mmoles/l, *i.e.* the optimum Mg^{++}/ATP ratio is now of the order of 2/1 (Fig. 6). A further increase of the

Mg^{++} concentration leads to a slight inhibition of the activity. Further addition of ATP results in a more marked inhibition of the activity.

The activation of the enzyme with $Na^{+} + K^{+}$ accordingly changes the optimum Mg^{++}/ATP ratio from 1/1 to 2/1.

Calcium: Ca^{++} inhibits the activity of the enzyme, and the inhibition in a system containing Mg^{++} or $Mg^{++} + Na^{+}$ or $Mg^{++} + K^{+}$ is different from that obtained in a system containing $Mg^{++} + Na^{+} + K^{+}$ (Fig. 7).

In the presence of Mg^{++} alone or $Mg^{++} + Na^{+}$ or $Mg^{++} + K^{+}$, *i.e.* when the optimum Mg^{++}/ATP ratio is 1/1, the Ca^{++} inhibition is accompanied by a decrease in the optimum Mg^{++} concentration.

In the presence of $Mg^{++} + Na^{+} + K^{+}$, *i.e.* when the optimum Mg^{++}/ATP ratio is 2/1, the Ca^{++} inhibition is accompanied by an increase in the optimum Mg^{++} concentration. The inhibition is counteracted by an increase in the Mg^{++} concentration, but the original activity is not fully restored even with high concentrations of Mg^{++}.

The two different ways in which Ca^{++} influences the optimum Mg^{++}/ATP ratio indicates two different types of Mg^{++} activation.

One type of activation is obtained in the presence of Mg^{++} alone or $Mg^{++} + Na^{+}$ or $Mg^{++} + K^{+}$; it is non-competitively inhibited by Ca^{++}, and the optimum Mg^{++}/ATP ratio is under these circumstances 1/1.—To this is, when the system contains $Mg^{++} + Na^{+} + K^{+}$, added another type of activation, which is inhibited competitively by Ca^{++}. The optimum Mg^{++}/ATP ratio is now 2/1.

ITP as substrate: In order to explore this hypothesis of two types of Mg^{++} activation, experiments were performed with ITP as substrate instead of ATP.

Studies of LEVEDAHL AND JAMES[7] on the rotatory dispersion of ATP seem to show that the ATP molecule is folded back upon itself, and that the NH_2 group of

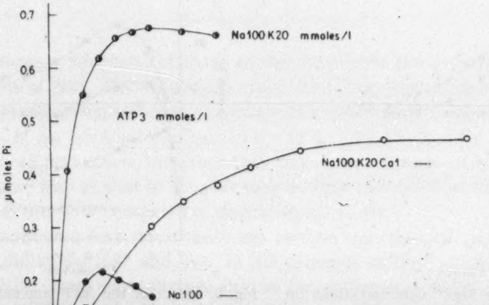


Fig. 7. Effect of Mg^{++} in the presence of Na^{+} and $Na^{+} + K^{+}$ and in the absence and presence of Ca^{++}. ATP concentration: 3 mmoles/l. Cations present: 100 mmoles/l Na^{+} + 0, 1 and 2 mmoles/l Ca^{++}, 100 mmoles/l Na^{+} + 20 mmoles/l K^{+} + 0 and 1 mmole/l Ca^{++}.

adenine together with the two terminal phosphate groups play a role in this folding. LEVEDAHL AND JAMES proposed that this folded structure is important for the activity of ATP in biological systems. SZENT-GYÖRGYI[8] has suggested that since Mg^{++} invariably seems necessary for the activity of ATP, it is this ion which connects the two ends of the folded molecule through a quadridentate chelate formation between the two terminal phosphate groups of the phosphate chain and the NH_2 group and the N7 group of the adenine part of the ATP molecule.

One or the other of the two effects of Mg^{++} on the Na^{+} + K^{+} activated ATPase may conceivably be due to such a chelate formation. In either case, one should expect to find a change of the optimum Mg^{++}/ATP ratio if ATP were replaced as substrate by ITP, which lacks the NH_2 group in the purine, but which is otherwise structurally identical with ATP.

The enzyme catalyses the hydrolysis of ITP to IDP + Pi, and also with ITP as substrate Mg^{++} is an obligatory requirement. It is observed that the activity with Mg^{++} alone is higher with ITP than with ATP as substrate (see Fig. 9) and that the optimum Mg^{++}/ITP ratio is 1/1.

In the presence of Mg^{++}, addition of one of the monovalent cations, Na^{+}, Li^{+}, K^{+}, Rb^{+}, Cs^{+} or NH_4^{+}, leads to an increase of the activity (Fig. 8), and it is noted, as in the experiments with ATP, that the increase is highest when the added monovalent cation is Na^{+} and lower with Li^{+} and the other monovalent cations. Contrary to what was found with ATP as substrate, the activating effect of Li^{+} and of the other monovalent cations is in these experiments almost the same. The increase of activity due to Na^{+} is smaller when the substrate is ITP than when it is ATP (Fig. 9); but since the activity due to Mg^{++} alone is higher with ITP as substrate, the total activity with Mg^{++} + Na^{+} becomes higher. The concentration of Na^{+} which gives

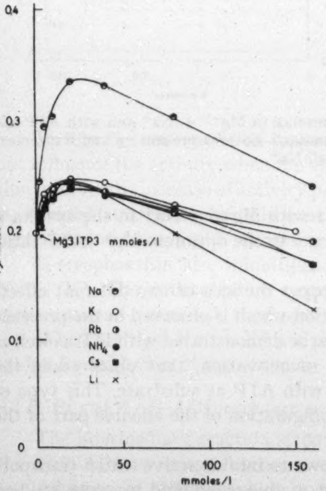


Fig. 8. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++} and with ITP as substrate. ITP concentration: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

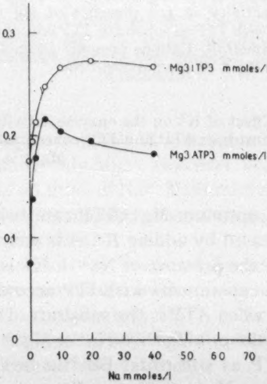


Fig. 9. Effect of Na^{+} on the enzyme activity in the presence of Mg^{++} and with ATP and ITP as substrate. ATP and ITP concentrations: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

half maximum activity with ITP as substrate is 2.5 mmoles/l compared to 1.4 mmoles/l with ATP as substrate. The optimum Mg^{++}/ITP ratio in the presence of $Mg^{++} + Na^{+}$ is 1/1.

When the system contains $Mg^{++} + Na^{+}$, the addition of one of the other monovalent cations, Li^{+}, K^{+}, Rb^{+}, Cs^{+} or NH_4^{+}, leads to an increase of the activity, and the effect is dependent on the Na^{+} concentration (Fig. 10). It should be noted that this stimulating effect is much smaller when ITP than when ATP is the substrate, and also that at a Na^{+} concentration of 100 mmoles/l the added cations lead to a decrease of the activity at lower concentrations than in the systems containing ATP as substrate. This is even more pronounced when the Na^{+} concentration is low, for example 20 mmoles/l (Fig. 11); in fact, under these circumstances K^{+} exerts only a very slight stimulating effect.

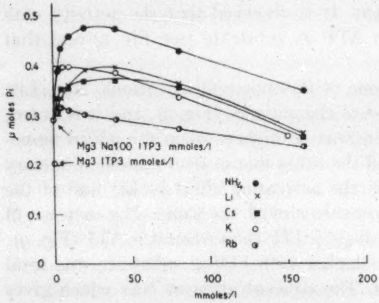


Fig. 10. Effect of monovalent cations on the enzyme activity in the presence of $Mg^{++} + Na^{+}$ and with ITP as substrate. ITP concentration: 3 mmoles/l. Cations present: 3 mmoles/l Mg^{++} + 100 mmoles/l Na^{+}.

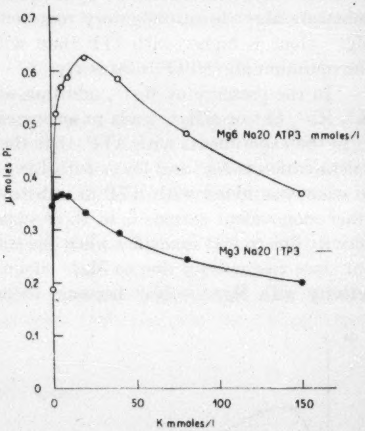


Fig. 11. Effect of K^{+} on the enzyme activity in the presence of $Mg^{++} + Na^{+}$ and with ATP and ITP as substrate. ATP and ITP concentrations: 3 mmoles/l. Cations present: 3 and 6 mmoles/l Mg^{++} + 20 mmoles/l Na^{+}.

The optimum Mg^{++}/ITP ratio which is 1/1 with $Mg^{++} + Na^{+}$ in the system is not increased by adding K^{+}, it is still 1/1, contrary to the optimum Mg^{++}/ATP ratio, which in the presence of $Na^{+} + K^{+}$ is 2/1 (Fig. 12).

The experiments with ITP accordingly support the idea of two different effects of Mg^{++} when ATP is the substrate. The activation which is observed in the presence of Mg^{++} alone or $Mg^{++} + Na^{+}$ or $Mg^{++} + K^{+}$ can be demonstrated with ITP as well as with ATP as substrate. But the second type of activation, that observed in the presence of $Mg^{++} + Na^{+} + K^{+}$, is found only with ATP as substrate. This type of activation must consequently depend on the configuration of the adenine part of the ATP molecule.

G-strophanthin: Since g-strophanthin is known to inhibit active cation transport in a number of biological systems[9,10], the effect of this compound was also studied (Fig. 13).

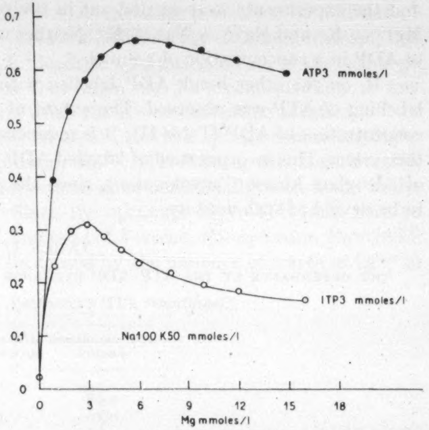


Fig. 12. Effect of Mg^{++} on the enzyme activity in the presence of Na^{+} + K^{+} and with ATP and ITP as substrate. ATP and ITP concentrations: 3 mmoles/l. Cations present: 100 mmoles/l Na^{+} + 50 mmoles/l K^{+}.

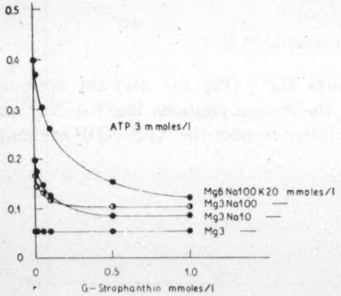


Fig. 13. Effect of g-strophanthin on the enzyme activity in the presence of Mg^{++}, Mg^{++} + Na^{+} and Mg^{++} + Na^{+} + K^{+}. ATP concentration: 3 mmoles/l. Cations present: 3 mmoles/l Mg^{++}; 3 mmoles/l Mg^{++} + 10 and 100 mmoles/l Na^{+} 6 mmoles/l Mg^{++} + 100 mmoles/l Na^{+} + 20 mmoles/l K^{+}.

Within the concentration range studied (up to 1 mmole/l), g-strophanthin does not influence the activity when the system contains only Mg^{++}. However, g-strophanthin inhibits the increase of activity produced by addition of Na^{+}. Maximum inhibition is obtained at a g-strophanthin concentration of 0.5 mmole/l; the inhibition is only partial and decreases with increasing Na^{+} concentration.

G-strophanthin also inhibits the increase of activity produced by addition of Na^{+} + K^{+}. The concentration required to give maximum inhibition is somewhat higher, around 1 mmole/l, than when the system contains only Mg^{++} + Na^{+}.

When Mg^{++} + Na^{+} + K^{+} are present in the system, addition of g-strophanthin leads to a reduction of the optimum Mg^{++}/ATP ratio from 2/1 to 1/1.

ATP–Pi and ATP–ADP exchange reactions

The intermediary reaction steps were investigated by studying the ATP–Pi and the ATP–ADP exchange reactions.

Addition of [^{32}P]phosphate to the system did not in any instance lead to a measurable labelling of ATP. The concentration of Pi varied from 1 to 10 mmoles/l, and the experiments were carried out in the presence of Mg^{++} alone, $Mg^{++} + Na^+$, $Mg^{++} + K^+$ and $Mg^{++} + Na^+ + K^+$. Neither were the results influenced by addition ot ADP in a concentration of 1 mmole/l.

If, on the other hand, ADP labelled with $AD^{32}P$ was added to the system, a labelling of ATP was observed. The extent of the labelling of ATP depends on the concentration of ADP (Table II); it is independent of whether or not Pi is added to the system. This incorporation of labelled ADP into ATP cannot be due to the action of adenylate kinase ("myokinase"), since the submicroscopic particles were shown to be devoid of such activity.

TABLE II

THE DEPENDANCE OF THE ATP–ADP EXCHANGE RATE ON THE ADP CONCENTRATION

Conditions: ATP 3 mmoles/l, Mg^{++} 3 mmoles/l.

ADP concentration mmoles/l	*ADP exchanged mμmoles/20 min*
0.08	40
0.16	116
1.16	260
2.66	238

The ATP–ADP exchange reaction requires Mg^{++} (Fig. 14), and the optimum Mg^{++}/ATP ratio is 1/1. This is so also when the system contains $Mg^{++} + Na^+$ and when it contains $Mg^{++} + Na^+ + K^+$; in the latter respect the ATP–ADP exchange reaction differs from the ATPase activity.

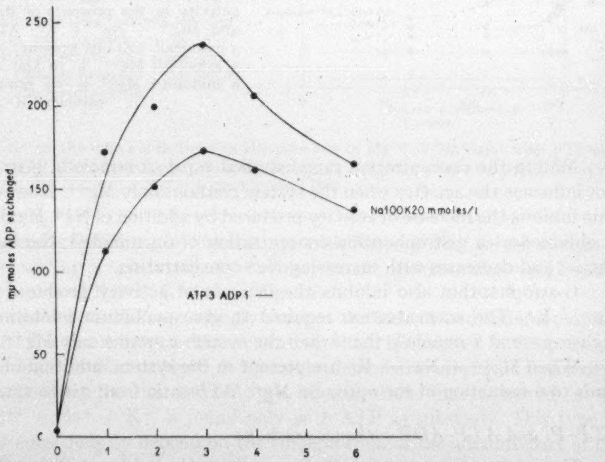


Fig. 14. Effect of Mg^{++} on rate of ATP–ADP exchange in the absence and presence of $Na^+ + K^+$. In Fig. 14–17 the exchange rate is expressed as mμmoles ADP exchanged per 20 min. ATP concentration: 3 mmoles/l. Cations present: 0 mmole/l Na^+ + 0 mmole/l K^+; 100 mmoles/l Na^+ + 20 mmoles/l K^+.

It appears from Fig. 15 that the ATP–ADP exchange is reduced by addition of Na^{+} to the system. The addition of K^{+} does not influence the exchange reaction, but in the presence of Na^{+} the addition of K^{+} leads to a further decrease of the exchange reaction (Fig. 16). It should be noted that when the Na^{+} concentration is low (10 mmoles/l), this inhibition disappears when the K^{+} concentration is raised.

Fig. 17 presents experiments on the effect of Ca^{++} in a system containing Mg^{++} alone. It may be noticed that the optimum Mg^{++}/ATP ratio in this experiment is somewhat lower than 1/1; similar observations have occasionally been made in determinations of the ATPase activity. Since the optimum Mg^{++}/ATP ratio may be raised somewhat by addition of small amounts of Versene, the optimum Mg^{++}/ATP ratio lower than 1/1 may presumably be caused by the presence of traces of Ca^{++} in the system.

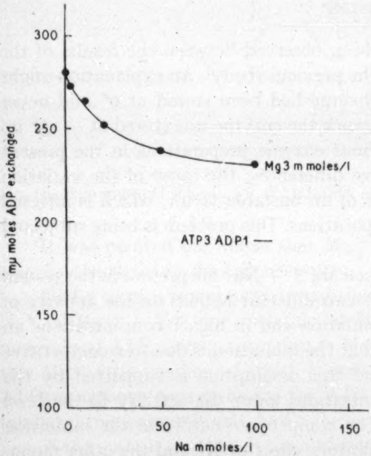


Fig. 15.

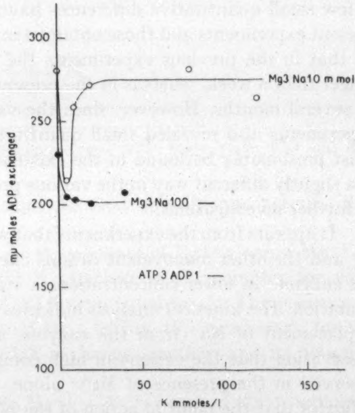


Fig. 16.

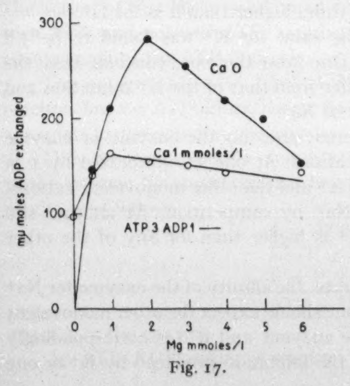


Fig. 17.

Fig. 15. Effect of Na^{+} on rate of ATP–ADP exchange in the presence of Mg^{++}. ATP concentration: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

Fig. 16. Effect of K^{+} on rate of ATP–ADP exchange in the presence of Mg^{++} + Na^{+}. ATP concentration: 3 mmoles/l. Cations present: 3 mmoles/l Mg^{++} + 10 and 100 mmoles/l Na^{+}.

Fig. 17. Effect of Mg^{++} on rate of ATP–ADP exchange in the absence and presence of Ca^{++}. ATP concentration: 3 mmoles/l. Cation present: 0 and 1 mmole/l Ca^{++}.

When Ca^{++} is added to the system, the exchange reaction is inhibited, and the optimum Mg^{++}/ATP ratio is markedly lowered. The effect of Ca^{++} on the exchange reaction thus corresponds to its effect on the ATPase activity when the optimum Mg^{++}/ATP ratio is 1/1.

G-strophanthin in the concentrations tested (up to 0.5 mmole/l), does not influence the ATP–ADP exchange reaction when the system contains only Mg^{++}, whereas in the presence of $Mg^{++} + Na^{+}$ or $Mg^{++} + Na^{+} + K^{+}$ the addition of g-strophanthin counteracts to some extent the inhibition due to Na^{+} or to $Na^{+} + K^{+}$.

The enzyme is also able to catalyse an exchange reaction between ITP and ADP; the rate of exchange amounts to 75–80 % of that between ATP and ADP. The ITP–ADP exchange also requires Mg^{++} and is slightly inhibited by Na^{+} and by $Na^{+} + K^{+}$.

DISCUSSION

A few small quantitative differences have been observed between the results of the present experiments and those obtained in the previous study[1]. An explanation might be that in the previous experiments the enzyme had been stored at 0° and never longer than a week, whereas in the present work the enzyme was stored at —15° up to several months. However, since the various enzyme preparations in the present experiments also revealed small quantitative differences, the cause of the variation must presumably be found in the existence of an unstable factor, which is affected in a slightly different way in the various preparations. This problem is being subjected to further investigations.

It appears from the experiments that when $Mg^{++} + Na^{+}$ are present in the system K^{+} and the other monovalent cations exert two different actions on the activity of the enzyme: in lower concentrations a stimulation and in higher concentrations an inhibition. The kinetical analysis indicates that the inhibition is due to a competitive displacement of Na^{+} from the enzyme, and this assumption is supported by the observation that the cations in high concentrations lower the activity to the level observed in the presence of Mg^{++} alone. The competitive nature of the inhibition indicates that the point of action of the inhibitory effect of K^{+} and the other monovalent cations is identical with the point of action of the activating effect of Na^{+}. The affinity for Na^{+} at this site is about 7–10 times higher than it is for K^{+}.

With $Mg^{++} + Na^{+}$ in the system the K_m value for K^{+} was found to be 1.8 mmoles/l and the K_I value to be 9 mmoles/l. One must therefore conclude that the point of action of the K^{+} stimulation must differ from that of the K^{+} inhibition and thereby also from that of the activating effect of Na^{+}.

There seem accordingly to be two different sites on the enzyme or enzyme complex with high affinities for monovalent cations. At one site (indicated by 1 in Fig. 18) the affinity for Na^{+} is higher than for K^{+} and the other monovalent cations, and at this site these cations can displace Na^{+} by competition. At another site (indicated by 2 in Fig. 18) the affinity for K^{+} is higher than for any of the other monovalent cations.

When ITP is substituted for ATP as substrate, the affinity of the enzyme for Na^{+} is lowered (Fig. 9). Under these circumstances one should expect the other monovalent cations to displace Na^{+} more readily from the enzyme, and it was correspondingly found that in a system with ITP as substrate the inhibition produced by K^{+} or one of the other monovalent cations is stronger and commences at lower concentrations than when the substrate is ATP. It should be noted, however, that the lower enzymic activity with ITP in the presence of $Na^+ + K^+$ is due, not only to a more pronounced K^+ inhibition, but also, and primarily, to a lower K^+ stimulation; this appears from experiments (not presented) in which Na^+ was present in sufficiently high concentration (350 mmoles/l) to counteract effectively the competitive K^+ inhibition.

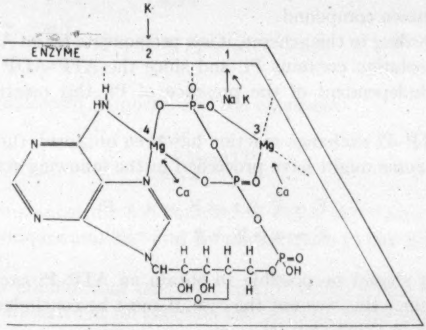


Fig. 18. Hypothetical scheme for the relation between enzyme, ATP, Mg^{++}, Ca^{++}, Na^+ and K^+.

It was pointed out above that Mg^{++} seems to exert two different actions; this may be visualized in the following way.

Under certain experimental conditions (in the presence of Mg^{++} alone or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$) one molecule of Mg^{++} is attached to the complex for each molecule of ATP, giving an optimum Mg^{++}/ATP ratio equal to 1/1. The presence of this Mg^{++} (the position of which is indicated by 3 in Fig. 18) is necessary for the ATPase activity as well as for the ATP–ADP exchange reaction, and its action is inhibited non-competitively by Ca^{++}.

If the system contains K^+ in addition to $Mg^{++} + Na^+$, a second Mg^{++} (indicated by 4 in Fig. 18) is added to the complex, giving an optimum Mg^{++}/ATP ratio of 2/1. The action of this Mg^{++} is inhibited competitively by Ca^{++}, and it is observed only with ATP, and not with ITP, as substrate, which shows that it is dependent on the adenine part of the ATP molecule.

The experiments with labelled ADP and Pi showed an ATP–ADP exchange reaction but no ATP–Pi exchange reaction. Since the preparation does not exhibit adenylate kinase activity, one may assume that an intermediate step in the breakdown of ATP is the formation of a phosphorylated enzyme in which the phosphate is bound to the enzyme by an energy-rich bond. The simplest scheme which can explain the results is as follows:

$$\begin{array}{ll} E + ATP \rightleftharpoons EATP & (1) \\ EATP \rightleftharpoons E \sim P + ADP & (2) \\ \underline{E \sim P + H_2O \rightarrow E + Pi} & (3) \\ ATP + H_2O \rightarrow ADP + Pi & \end{array}$$

where E is the enzyme.

An ATP–ADP exchange reaction might also have been obtained if, instead of a phosphorylated enzyme, an $E \sim ADP$ were formed, but in this case the reaction should proceed as follows[11]:

$$E + ATP \rightleftharpoons EATP \tag{4}$$

$$EATP \rightleftharpoons E \sim ADP + Pi \tag{5}$$

$$E \sim ADP + x \rightleftharpoons E \sim x + ADP \tag{6}$$

where x is an unknown compound.

However, according to this scheme, it is a prerequisite for an ATP–ADP exchange reaction that the solution contains Pi, and since the ATP–ADP exchange reaction was found to be independent of the presence of Pi, this reaction scheme can be disregarded.

Even if no ATP–Pi exchange reaction has been obtained, the breakdown of the phosphorylated enzyme might have proceeded in the following way[11]:

$$E \sim P + x \rightleftharpoons E \sim x + Pi \tag{7}$$

$$E \sim x \rightarrow E + x \tag{8}$$

In this case it should be possible to obtain an ATP–Pi exchange reaction by adding ADP, and since this was not the case, it must be concluded that the reaction proceeded as shown in expression (3).

In all systems capable of catalysing an ATP–ADP exchange reaction Mg^{++} has been found to be an obligatory requirement[11–13], and it has also been found that the optimum Mg^{++} concentration is equal to the concentration of ATP[13]. This has been confirmed in the present experiments. No evidence is available as to how this Mg^{++} exerts its effect, but the 1/1 ratio between Mg^{++} and ATP suggests that it is involved in the formation of the enzyme–substrate complex.

Na^+ has a moderate inhibitory effect on the ATP–ADP exchange reaction, whereas K^+ is without effect. With $Mg^{++} + Na^+$ present in the system, addition of K^+ gives rise to two different effects. A low concentration of K^+ relative to that of Na^+ leads to an intensification of the Na^+ inhibition, while a K^+ concentration which is high relative to the Na^+ concentration restores the rate of the reaction which was observed in the presence of Mg^{++} alone.

There is thus a qualitative similarity between the effects of K^+ on the ATPase activity and on the exchange reaction. In both cases the effect is dependent on the presence of Na^+, and in both cases high concentrations of K^+ relative to Na^+ abolishes the effect. In experiments on the ATPase activity the latter phenomenon has been found to be due to a displacement of Na^+, and the same mechanism appears likely also in the case of the exchange reaction. This similarity between the K^+ effects indicates that it is the same enzyme which is responsible for the hydrolysis of ATP and for the ATP–ADP exchange reaction.

Na^+ and, still more pronounced, $Na^+ + K^+ +$ an extra molecule of Mg^{++} increase the liberation of Pi from ATP, *i.e.* step (3) of the reaction. It is important to note, however, that according to the experiments with ITP the configuration of the adenine part of the ATP molecule is of importance for the activating effect of $Na^+ + K^+ + Mg^{++}$, and it must consequently be assumed that the binding of the cations to the enzyme takes place already during the attachment of ATP to the enzyme, *i.e.* already during process (1).

The reaction sequence seems accordingly to be as follows:

$$\text{E} + \text{ATP} + 2\,\text{Mg} + n\text{Na} + m\text{K} \rightleftharpoons \underset{n\text{Na}}{\overset{m\text{K}}{\text{EMg}_2\text{ATP}}} \tag{9}$$

$$\underset{n\text{Na}}{\overset{m\text{K}}{\text{EMg}_2\text{ATP}}} \rightleftharpoons \underset{n\text{Na}}{\overset{m\text{K}}{\text{E}}} \sim \text{P} + 2\,\text{Mg} + \text{ADP} \tag{10}$$

$$\underset{n\text{Na}}{\overset{m\text{K}}{\text{E}}} \sim \text{P} \rightarrow \text{E} + \text{Pi} + n\text{Na} + m\text{K} \tag{11}$$

where n and m are unknown numbers, and the expression

$$\underset{n\text{Na}}{\overset{m\text{K}}{\text{EMg}_2\text{ATP}}}$$

symbolises an enzyme–substrate–ion complex in which Mg^{++} constitutes part of the enzyme–substrate complex and Na^{+} and K^{+} are attached to two different sites on the enzyme (see Fig. 18).

G-strophanthin inhibits that part of step (11) which is due to the presence of Na^{+} or $Na^{+} + K^{+}$, while it has no effect on the splitting of the phosphorylated compound which is obtained with Mg^{++} alone. With $Na^{+} + K^{+}$ in the system the inhibition is accompanied by a decrease of the optimum Mg^{++}/ATP ratio from 2/1 to 1/1. Furthermore, g-strophanthin counteracts to some extent the inhibition of the exchange reaction due to Na^{+} or $Na^{+} + K^{+}$. The effect of g-strophanthin seems accordingly to be due to an inhibition of the binding of Na^{+} and K^{+} to the enzyme in step (9) and thereby indirectly to an inhibition of that part of step (11) which is due to Na^{+} or $Na^{+} + K^{+}$.

The 1/1 ratio of Mg^{++}/ATP suggests that Mg^{++} is involved in the formation of the enzyme substrate complex. It seems possible that Mg^{++} (3 in Fig. 18), in analogy with SMITH'S[14] theory for the role of metal ions for the peptidase activity, by a chelate formation between the two terminal phosphate groups and groups in the enzyme binds ATP to the enzyme. Mg^{++} acts as an electronic bridge and is thereby able to establish a connection between the electronic system in the phosphate chain and that in the protein. A displacement of electrons is hereby made possible, so that the P–O–P bond is weakened with the resultant possibility of hydrolytic splitting.

It is tempting to assume that the second Mg^{++}, which is involved in the activating effect of $Na^{+} + K^{+}$ and the effect of which is dependent on the NH_2 group in the adenine part of ATP, becomes attached to the ATP molecule as suggested by SZENT-GYÖRGYI[8] (4 in Fig. 18). As pointed out by SZENT-GYÖRGYI, such a chelate formation will make one common electronic system of the phosphate chain and of the adenine with common non-localised electrons which could transport energy.

This model with two Mg^{++} ions in the enzyme-ATP complex opens the possibility that this common electronic system of ATP may, through the Mg^{++} placed as shown in position 3 in Fig. 18 be connected with and thereby able to act on the electronic system of the enzyme. It may be through such an interaction of the two electronic systems that the enzyme develops the peculiar and specific affinities for Na^{+} and K^{+}.

In the previous study it was pointed out that the enzyme investigated possesses properties that make it reasonable to assume that it is involved in the active transport of cations across the nerve membrane. The present experiments seem to lend further support to this idea.

An enzyme involved in transport across the nerve membrane must be expected to be located in the membrane, and although no direct evidence is available on this point concerning the enzyme studied, various observations may indicate that in the intact nerve it is, in fact, located here. LIBET[15] and ABOOD AND GERARD[16] investigated an ATPase in peripheral nerves which, like the one studied here, was activated by Mg^{++} and which after homogenisation and differential centrifugation was located in submicroscopic particles. This enzyme could be isolated from the sheath but not from the axoplasm of giant axons. Furthermore, HANZON AND TOSCHI[17] studied by the electron microscope a submicroscopic particle fraction from nerve tissue, isolated under conditions similar to those used here, and found that the appearance of these particles indicated that they were fragments of disintegrated membrane.

HODGKIN AND KEYNES[18] have demonstrated that in peripheral nerve the outward transport of Na^+ is linked to a transport of K^+ into the fibre. One might therefore expect an enzyme involved in this transport to have a high affinity for both Na^+ and K^+, and one might also expect that the location of these affinities differed from one another. As appears from the experiments reported above, these are the properties shown for the enzyme studied.

One may visualize an arrangement of the system in the membrane according to which the site of the Na^+ affinity faces the intracellular water phase, while the site of the K^+ affinity is in contact with the extracellular water phase. At the site with highest Na^+ affinity the saturation of the enzyme with Na^+ depends on both the Na^+ and the K^+ concentration, and it appears from Fig. 9 in the previously published paper that the Na^+ saturation is about 40 % when the Na^+ and K^+ concentrations are 40 and 350 mmoles/l, respectively, *i.e.* identical with the intracellular concentrations of these ions in crab nerve. Since the activity varies with the Na^+ saturation, a change in Na^+ concentration above and below that corresponding to the intracellular level must consequently lead to a change in the enzyme activity. Moreover, due to the competition between Na^+ and K^+ a decrease in the K^+ concentration will increase the activity of the enzyme, while an increase in the concentration of K^+ will decrease the activity.

At the site with highest K^+ affinity the saturation with K^+ is equal to 80–90 % when the K^+ concentration is around 12 mmoles/l, *i.e.* identical with the extracellular K^+ concentration in the crab.

The enzyme has accordingly such affinities for Na^+ and K^+ as would be appropriate if the function of the enzyme were to control the magnitude of and the ratio between the intracellular Na^+ and K^+ concentrations.

It may finally be mentioned that the enzyme activity due to Na^+ and $Na^+ + K^+$ is inhibited by g-strophanthin. No data are available concerning the effect of g-strophanthin on active cation transport across the nerve membrane, but it is known that g-strophanthin inhibits the active cation transport in red blood cells[9] and in frog skin[10].

REFERENCES

[1] J. C. SKOU, *Biochim. Biophys. Acta*, 23 (1957) 394.
[2] R. L. POST, *Federation Proc.*, 18 (1959) 121.
[3] C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 224 (1957) 561.

[4] W. E. COHN in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Academic Press, New York, Vol. III, 1957, p. 867.
[5] H. M. KALCKAR, *J. Biol. Chem.*, 167 (1947) 461.
[6] J. S. FRIEDENWALD AND G. D. MAENGWYN-DAVIES, *Symposium on the Mechanism of Enzyme Action*, The John Hopkins Press, Baltimore, 1954 p. 154.
[7] B. H. LEVEDAHL AND T. W. JAMES, *Biochim. Biophys. Acta*, 21 (1956) 298.
[8] A. SZENT-GYÖRGYI, *Bioenergetics*, Academic Press, New York, 1957.
[9] H. J. SCHATZMANN, *Helv. Physiol. Acta*, 11 (1953) 346.
[10] V. KOEFOED-JOHNSEN, *Acta Physiol. Scandinav.*, 42 *suppl.*, 145 (1957) 87.
[11] C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 233 (1958) 1589.
[12] G. ULBRECHT AND M. ULBRECHT, *Biochim. Biophys. Acta*, 29 (1957) 100.
[13] J. R. BRONK AND W. W. KIELLY, *Biochim. Biophys. Acta*, 29 (1958) 369.
[14] E. L. SMITH, *Advances in Enzymology*, 12 (1951) 191.
[15] B. LIBET, *Federation Proc.*, 7 (1948) 72.
[16] L. G. ABOOD AND R. W. GERARD, *J. Cellular Comp. Physiol.*, 43 (1954) 379.
[17] V. HANZON AND G. TOSCHI, *Exptl. Cell. Research*, 16 (1959) 256.
[18] A. L. HODGKIN AND R. D. KEYNES, *J. Physiol.*, (*London*), 128 (1955) 28.

A TECHNIQUE FOR ELUTION OF PROTEINS FROM STARCH GEL

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SUMMARY

A technique for recovery of separated plasma proteins after electrophoresis in starch gel is described. By means of a second electrophoresis applied to sections which have been cut from the main gel almost quantitative recoveries of these proteins, except for γ-globulin were obtained. The trace amounts of protein originating from the gel itself under these conditions were measured. Considerable amounts of starch are eluted at the same time but do not interfere with the estimation of the plasma proteins.

INTRODUCTION

With the increasing use of electrophoresis in gels the need has become apparent for a simple and quantitative method for recovery of the separated materials. A technique involving lifting an agar gel on to a cellophane membrane placed so as to isolate pools of buffer at anode and cathode ends of the slab followed by a period of elution by electrophoresis into these pools has been described[1]. Since the introduction of electrophoresis in starch gel by SMITHIES[2], several methods for elution from such gels have been worked out. That of JARRIGE AND LAFOSCADE[3] depends on cutting a slot in the gel in front of the band of material to be eluted, filling this with buffer and cotton wool and eluting by electrophoresis into the buffer. MORETTI, BOUSSIER AND JAYLE[4] on the other hand, preferred to cut out the piece of gel containing the material under investigation and elute from it after fragmentation. Since the SMITHIES[2] method for electrophoresis of plasma proteins is now in very wide use, a simpler apparatus than that used by MORETTI, BOUSSIER AND JAYLE has been designed. Using this apparatus conditions for recovery in good yield of all the proteins present in rat serum, with the exception of γ-globulin, have been worked out.

The principle of the new apparatus is that the piece of gel to be eluted has placed on its upper surface a vertical rectangular slab of the same gel containing only dilute buffer solution. This upper slab of gel has the same cross sectional area as the piece of gel to be eluted and, because of its excellent mechanical properties, does not require any support. Passage of current through the gel causes migration of protein from the bottom section into a small volume of buffer solution placed immediately below it. This buffer solution is separated from a large volume of the same solution by means of a cellophane membrane and the eluted protein is thus trapped in the small volume. During elution the gel rests in the upper part of a vertical channel cut through three rectangular pieces of perspex (Fig. 2A) which are screwed tightly together to form a single unit. Support for the gel is provided by several narrow polythene bridges stretching across the channel and forming part of a sheet of this material which is held between the two uppermost pieces of perspex. The upper perspex rectangle is of the same thickness as is the gel to be eluted. The thickness of the middle section (6 mm in the present apparatus) determines the volume of buffer into which the protein will migrate. The cellophane membrane is held between the

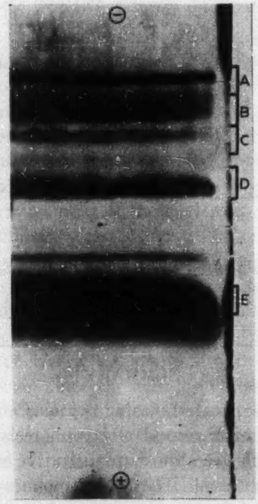

Fig. 1. Pattern of plasma proteins obtained by electrophoresis of 0.15 ml of rat serum for 20 h at 4.0 V/cm.

two lower-most pieces of perspex. As shown in Fig. 2B cross bars are screwed to the top of the perspex rectangles so that the unit can rest on the top of a large filtrate jar. Contact from the top surface of the upper slab of gel is made with a horizontal slab of the same material which in turn is kept in electrical contact with the buffer solution in the cathode vessel by means of wet folded muslin.

In preliminary experiments 0.02 *M* borate buffer was used both in the gel above the section being eluted and for the solution in the electrode vessels. The same buffer had been used for the original electrophoresis and it was thought likely that the gel would best retain its shape if there was no discontinuity either in the type of buffer ion present or in concentration. In practice, however, a sharp fall in pH occurred at the buffer–gel interface (the lower surface of the piece of gel being eluted). As the region of decreased pH moved upwards the gel became distorted. Furthermore, since the fall of pH was of several units, very poor recoveries of protein were obtained. Fortunately, however, no effect of this kind took place when phosphate ions were used instead of borate. The reason for the decrease in pH of part of the gel which occurs in presence of borate ions is not understood but may be connected with the ability of these ions to complex with starch. Satisfactory recoveries were obtained with 0.02 *M* phosphate buffer at pH 8.3 in the gel bridge and in the electrode vessels. The small amount of borate ions in the section of gel being eluted, compared with the

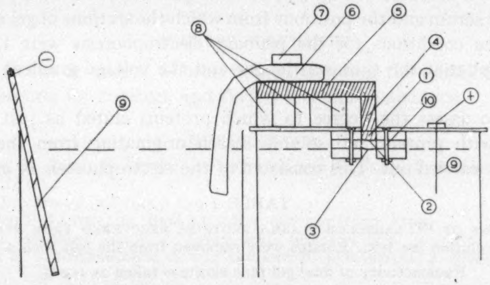

Fig. 2a.

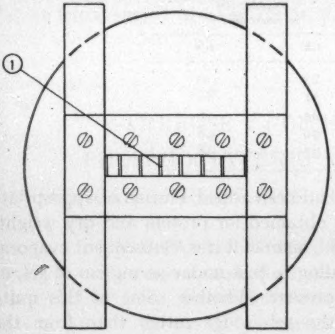

Fig. 2b.

Fig. 2. A. Complete apparatus with elution cell resting on top of a 3-l filtrate jar. Only the upper part of this is shown. 1. Section of gel being eluted. 2. Polythene bridges. 3. Cellophane membrane. 4. Vertical rectangular slab of starch gel (starch pillar). 5. Horizontal slab of starch gel. 6. Wet folded muslin. 7. Glass weight. 8. Perspex supports for gel bridge. 9. Platinum electrodes. 10. Brass screws. B. Plan of elution cell showing polythene bridges (1) which support the piece of gel during elution.

total amount of phosphate ions in the rest of the gel, is insufficient to prevent an adequate degree of elution. At 300 V the current of 58 mA caused only slight warming of the gel bridge. During electrophoresis the volume of solution into which the protein was being eluted was found to increase slightly. To prevent overflow small amounts were occasionally sucked off from the angle between the upper slab of gel and the surface of the perspex rectangle and kept for estimation with the final eluate. The proportions of protein eluted under these conditions from various sections of the gel are shown in Table I. Fig. 1 shows the pattern obtained by electrophoresis of 0.15 ml of rat serum and the positions from which the sections of gel used for elution were taken. The conditions for the primary electrophoresis were those given by SMITHIES[2] except that the time was longer and the voltage gradient was lower (*cf.* Fig. 1).

In order to assess the degree to which proteins eluted as just described are contaminated with protein and soluble starch originating from the gel, a blank experiment was carried out. This consisted of the electrophoresis of a slab of starch 7 cm wide and 0.65 cm deep to which nothing had been added, elution of appropriate sections, and examination of the eluates thus obtained for protein and dry weight. Before analysis the eluates were dialysed for 48 h against water. Subsequent evaporation to dryness revealed dry weight corresponding to just under 30 mg/cm of gel, or approximately 5 % of the starch originally present. Whether some of this quite considerable quantity of starch came from the gel bridge rather than from the actual piece of gel undergoing elution was next investigated. For this purpose an elution was done with a rectangle of cellophane membrane interposed between the section being treated and the starch pillar resting on it. Under these conditions the proportion of soluble starch in the eluate dropped to 2.5 %. The amounts of material reacting as protein by the method of LOWRY *et al.*[5] in eluates made from the blank gel, with and without the interposed cellophane, were also estimated. No effect due to the cellophane could, however, be detected since both eluates were found to contain just under 50 μg protein.

TABLE I

PROPORTIONS OF ^{131}I-LABELLED PLASMA PROTEINS RECOVERED FROM STARCH GEL

For conditions of elution see text. Eluates were removed from the cell with a Pasteur pipette. Radioactivity of final gel plus eluate is taken as 100 %.

Section of Starch		*% radioactivity in eluate after*	
	cm from origin	*1 h*	*2 h*
A	2.5–3.5	23	70
B	3.5–4.5	31	86
C	4.5–5.5	64	96
D	6.0–7.0	96	98
E	10.0–11.0	95	98

In another experiment 0.15 ml of rat plasma was applied to the gel. Elution of a section corresponding to cut D in Fig. 1 which was at the same distance from the start as had been the blank section above, gave 950 μg protein by the method of LOWRY *et al.*[5] before correction for the blank. Since in this case the blank value to be subtracted was only just over 5 % of the total protein eluted, correction can evidently be made without noticeable loss of accuracy.

Since, however, cut D in Fig. 1 contains one of the stronger bands, larger amounts of plasma would need to be applied to the gel for accurate estimations in this way of the weaker bands. Fortunately, however, certain estimations such as immunological tests in gels[6] or scintillation counting for radioactivity are unaffected both by the considerable quantities of soluble starch or the much lower amounts of protein derived from the gel which are present in the eluates made as described above. If necessary soluble starch can be eliminated from the eluate by chromatography on Dowex-2 by the method of DE PAILLERETS *et al.*[7].

ACKNOWLEDGEMENTS

I am grateful to Dr. S. COHEN who carried out some of the initial experiments and to Mr. L. ASSEMAKIS for criticism and skilled technical assistance.

REFERENCES

[1] A. H. GORDON, B. KEIL, K. ŠEBESTA, O. KNESSL AND F. ŠORM, *Collection Czechoslov. Chem. Commun.*, 15 (1950) 1.

[2] O. SMITHIES, *Biochem. J.*, 61 (1955) 629.

[3] P. JARRIGE AND P. LAFOSCADE, *Bull. soc. chim. biol.*, 40 (1959) 1197.

[4] J. MORETTI, G. BOUSSIER AND M. F. JAYLE, *Bull. soc. chim. biol.*, 40 (1958) 59.

[5] O. H. LOWRY, N. J. ROSEBROUGH, A. C. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

[6] Ö. OUCHTERLONY, *Acta Path. Microbiol. Scand.*, 32 (1953) 231.

[7] C. DE PAILLERETS, J. MORETTI AND M. F. JAYLE, *Bull. soc. chim. biol.*, 41 (1959) 1285.

D-THREOSE 2,4-DIPHOSPHATE INHIBITION OF 3-PHOSPHOGLYCERIC ACID PHOTOREDUCTION BY A SONICALLY RUPTURED SPINACH CHLOROPLAST SYSTEM

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SUMMARY

1. [1-^{14}C]PGA is readily reduced by a sonically ruptured chloroplast system in light. This reduction is inhibited by threose 2,4-diphosphate.

2. PGA accumulates when $^{14}CO_2$ is incubated in light with the sonically ruptured chloroplast system in the presence of threose 2, 4-diphosphate. This indicates that PGA is in the major pathway of photosynthetic CO_2 fixation in the system.

3. Threose diphosphate inhibition of the total $^{14}CO_2$ fixation by the carbon cycle of sonically ruptured spinach chloroplasts is not due to inhibition of carboxydismutase, but due primarily to inhibition of triose phosphate dehydrogenase which in turn limits the formation rate of the photosynthetic carbon cycle CO_2 acceptor, RuDP.

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INTRODUCTION

The first stable intermediate formed by $^{14}CO_2$ fixation in illuminated photosynthetic systems is PGA[1]. Recently, an alternative path has been suggested for photosynthetic CO_2 fixation which does not involve PGA.[2] In this suggested path, carboxylation of RuDP yields a six carbon branched chain acid which subsequently rearranges without cleavage to PGA, to form a six carbon chain directly. If it were possible to block the reduction of PGA to triose in the photosynthetic carbon cycle, then it should be possible to observe whether CO_2 still flows into the cycle via some pathway other than that involving PGA.

Recently threose 2,4-diphosphate was shown to inhibit specifically muscle and yeast triose phosphate dehydrogenase having DPN as a cofactor[3,4]. This inhibitor has a K_i of $2 \cdot 10^{-7}$ for the oxidation reaction. The reduction reaction is less sensitive to the inhibitor, a concentration of about $2 \cdot 10^{-5}$ M yielding 50% inhibition. The inhibition of the enzyme is apparently due to the similarity of the threose diphosphate and 1,3-diphosphoglyceric acid molecules. It was of interest to determine whether threose diphosphate would also inhibit triose phosphate dehydrogenase in a TPN requiring photosynthetic system. Such an inhibition would then make it possible for PGA to accumulate when $^{14}CO_2$ was photosynthetically fixed in the presence of threose diphosphate. Since the photosynthetic carbon cycle involves a number of enzymic steps other than triose phosphate dehydrogenase, it was of interest to find whether some of these other enzymes were also inhibited by threose diphosphate. The experiments reported in this paper were done with a spinach chloroplast system since phosphorylated intermediates such as PGA and threose diphosphate do not readily penetrate intact cells. Whole chloroplasts were also found to be somewhat impermeable to the phosphorylated substrates and cofactors used in these experiments, confirming the observations of TOLBERT[5]. We found that sonic rupture of whole chloroplasts removed the permeability barrier and increased the rate of CO_2 fixation on a unit chlorophyll basis. Sonically ruptured chloroplasts proved more suitable for these experiments than either whole or broken chloroplasts as prepared by ARNON[6].

PROCEDURES

Whole chloroplasts were prepared from the leaves of *Spinacea oleracea* by grinding in 0,5 M sucrose buffer and were purified by fractional centrifugation according to the method described by HOLM-HANSEN *et al.*[7]. The whole, washed chloroplasts were resuspended in about 20 times their own volume of 10^{-3} M potassium phosphate buffer, pH 7.4. The chloroplast suspension was then sonically ruptured at 0° for 90 sec in a 9 kc, 100 W Raytheon magnetostriction oscillator operated at full voltage. The sonically ruptured suspension was used directly in the experiments. Investigation of this material with the light microscope showed no intact chloroplasts, and only a few large chloroplast fragments. Chlorophyll concentrations in the sonically ruptured suspension were determined spectrophotometrically in 80% acetone according to ARNON[8].

Abbreviations used: ADP, adenosine diphosphate; GSH, glutathione; HOAc, acetic acid; PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceric acid; RuDP, ribulose 1,5-diphosphate; SAS, saturated ammonium sulfate; TPN, triphosphopyridine nucleotide; TPP, thiamine pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; DPN, diphosphopyridine nucleotide.

The organic cofactors and metal cofactors were prepared separately and titrated to pH 7,4. The photosynthetic reactions were run at 24° and 1000 foot candles in airtight 35-ml flasks. The total reaction mixture volume was about 1.5 ml and contained about 1 mg of chlorophyll.

After 30-min incubation the enzymic reaction was stopped by addition of 4 ml of ethanol to the reaction mixture. The precipitate was then extracted with 20% ethanol, 100% ethanol and water successively. The extracts were pooled giving a total volume of ~ 10 ml. A small aliquot was acidified and placed on an aluminum planchet for assay of the total radioactivity in the reaction mixture. Another aliquot was applied to the origin of a paper chromatogram.

Two dimensional chromatography of the photosynthetic products and counting of the radioactive areas on the chromatograms was carried out according to the methods described by BASSHAM and CALVIN[1].

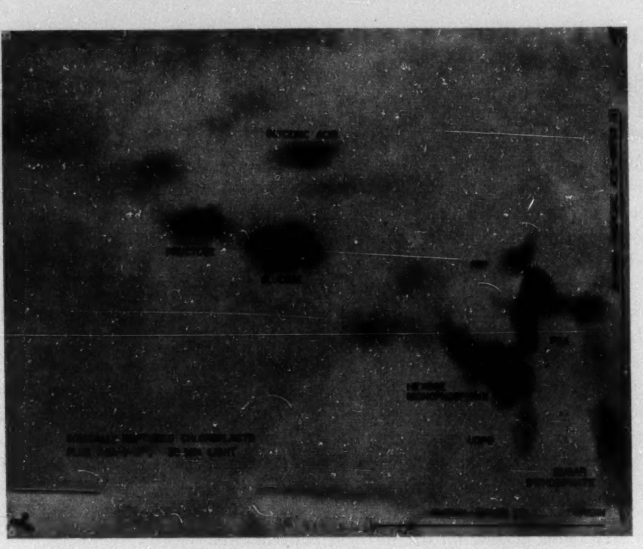
Carboxydismutase was prepared from *Tetragonia expansa* chloroplasts. These were prepared in 0.5 *M* sucrose and suspended in dilute buffer according to the method of SOGO *et al.*[9]. Carboxydismutase was obtained by ammonium sulfate fractionation of the clear supernatant (termed "chloroplast extract" in ref. 9) obtained from centrifugation of the chloroplast suspension. The procedure of MAYAUDON[10], normally used for the partitial purification of the crude extract of *Tetragonia expansa*, was modified for fractionation of the chloroplast extract, the material precipitating between 0.34 to 0.49 SAS being chosen for use in these experiments.

Carboxyl-labeled PGA was prepared by incubating RuDP with $NaH^{14}CO_3$ in the presence of carboxydismutase[11] and Mg^{++} (see ref. 12, 13). The reaction mixture consisted of 12.7 μM $NaH^{14}CO_3$ (300μC), 120 μM $MgCl_2$, 900 μM Tris at pH 8.3, approx. 3 μM RuDP, and 1.4 mg of enzyme preparation. The final volume of the incubation mixture was 2.25 ml. Incubation was carried out at room temperature for 20 h. The yield of radioactive PGA was about 1.5 μM, or 50 % of the theoretical value. The [1-^{14}C]PGA was purified by chromatography on washed No. 4 Whatman paper using a butanol–propionic acid–water system. The [^{14}C]PGA was located by radioautography and was recovered by elution. The eluate was taken to dryness and redissolved in 0.5 ml of 0.0402 *N* $NaH^{12}CO_3$.

D-threose 2,4-diphosphate was generously provided by Dr. BALLOU and Dr. FLUHARTY of the University of California Biochemistry Department.

EXPERIMENTAL RESULTS AND DISCUSSION

Preliminary experiments were done in which [1-^{14}C]PGA was incubated with the sonically ruptured chloroplast system in light and dark with the cofactors given in Table I. Chromatograms from this experiment (Fig. 1) showed that reduction of [1-^{14}C]PGA to triose phosphate in this enzyme system is a light dependent process. In the dark [1-^{14}C]PGA is not reduced. This accounts for the relatively large amounts of glyceric acid and alanine in the dark experiment. When [1-^{14}C]PGA reduction was established as a light dependent process in the system, threose diphosphate inhibition of spinach chloroplast triose phosphate dehydrogenase was investigated by incubating [1-^{14}C]PGA with sonically ruptured spinach chloroplasts in light in the presence and absence of threose 2,4-diphosphate. The reaction mixtures for this experiment are given in Table I. After two dimensional paper chromatography of the reaction mixture extract



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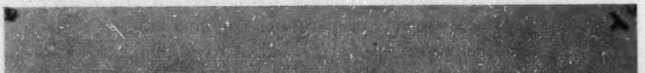

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Fig. 1. Chromatograms of extracts from sonically ruptured spinach chloroplast systems incubated with [1-^{14}C]PGA in light and dark.

TABLE I

DISTRIBUTION OF ^{14}C AMONG PGA, THE MONOPHOSPHATE SUGARS AND DIPHOSPHATE SUGARS WHEN [1-^{14}C]PGA IS INCUBATED WITH AND WITHOUT THREOSE DIPHOSPHATE IN AN ILLUMINATED CHLOROPLAST SYSTEM

Each reaction mixture contained (amount in μM) $MgCl_2$ 4.0, $MnSO_4$ 1.6, ADP 1.0, TPN 0.02, TPP 1.4, ascorbate 2.5, GSH 1.3, $NaH^{12}CO_3$ 4.0, fragmented chloroplasts 1 ml, [1-^{14}C]PGA 1,200,000 counts/min or approx. 0.1 μM. Threose 2,4-diphosphate 0.12 (present only in one reaction mixture). Total volume 1.275 ml.

	Counts/min on chromatogram	
	— Threose diphosphate	*+ Threose diphosphate*
Total	27,000	23,000
PGA	5,800	12,100
Monophosphates	7,300	1,900
Diphosphates	3,100	800

and location of the ^{14}C containing spots by autoradiography, the activity of the PGA area was compared with the activity of mono- and diphosphate sugar areas. The results of this experiment are given in Table I. The presence of 10^{-4} M threose diphosphate increased the ratio of PGA activity to the activity of the mono- and diphosphate sugar areas which would be expected if triose phosphate dehydrogenase were inhibited during operation of the photosynthetic cycle.

A second experiment was done in which $^{14}CO_2$ fixation was studied in the presence and absence of threose diphosphate. The reaction mixtures were identical to those given in Table I except that 4 μM $NaH^{14}CO_3$ (100 μC) were substituted for the [1-^{14}C] PGA. The results of this experiment ware given in Table II. Again there is an increase in the ratio of PGA activity to phosphorylated sugar activity in the threose diphosphate inhibited system.

The similarity between chromatograms of extracts from $H^{14}CO_3^-$ and [1-^{14}C]PGA incubated systems indicates that PGA is readily assimilated into the $^{14}CO_2$ pathway

TABLE II

DISTRIBUTION OF ^{14}C AMONG PGA, THE MONOPHOSPHATE SUGARS AND DIPHOSPHATE SUGARS WHEN $H^{14}CO_3^-$ IS INCUBATED WITH AND WITHOUT THREOSE 2,4-DIPHOSPHATE IN AN ILLUMINATED CHLOROPLAST SYSTEM

Each reaction mixture contained (amount in μM) $MgCl_2$ 4.0, $MnSO_4$ 1.6, ADP 1.0, TPN 0.02, TPP 1.4, ascorbate 2.5, GSH 1.3, $NaH^{14}CO_3$ 4.0(100 μC), sonically ruptured chloroplasts 1 ml.

	Counts/min on chromatogram per aliquot	
	— Threose diphosphate	*+ Threose diphosphate*
Total	230,000	98,000
PGA	93,000	60,000
Monophosphates	53,000	8,200
Diphosphates	23,000	7,000
	Total counts fixed in the reaction mixture	
Light	16,000,000	5,800,000
Dark	130,000	110,000

of the photosynthetic carbon cycle. The qualitative and quantitative similarity in the effect of threose diphosphate inhibition of the two systems further indicates that PGA is a real and major intermediate in the photosynthetic carbon cycle. While these experiments show that $^{14}CO_2$ photosynthetically fixed by a chloroplast system flows through PGA, they do not settle the question of whether the other three carbon fragment resulting from cleavage of the RuDP carboxylation product is also PGA[14].

An interesting feature of Table II is that the light fixation of $^{14}CO_2$ in the presence of threose diphosphate is about one-third of the control fixation. Inhibition of any of the enzymic steps of the photosynthetic carbon cycle to a rate limiting level would be expected to reduce the total $^{14}CO_2$ fixation by the cycle. The location of threose diphosphate inhibition in the photosynthetic carbon cycle of spinach chloroplast fragments is shown in Table I to be at least in part at triose phosphate dehydrogenase. There is the possibility that 10^{-4} M threose diphosphate may also be affecting the rates of other photosynthetic carbon cycle enzymes. Inhibition of most of these enzymes would limit the rate of formation of the acceptor molecule for CO_2 fixation, RuDP. Inhibition of carboxydismutase, however, could also involve direct inhibition of $^{14}CO_2$ entry into the cycle rather than just limitation of ribulose diphosphate formation.

It was of interest for this reason to test the effect of threose diphosphate on carboxydismutase activity both in the crude spinach preparation and in a purified enzyme system isolated from *Tetragonia expansa*. The reaction mixtures and results for these experiments are given in Fig. 2 and Table III. These data show that 10^{-4} M threose diphosphate has no effect on the carboxydismutase of the crude system, and inhibits the purified system, by only 15 %.

This result indicates that 10^{-4} M threose diphosphate inhibition of $^{14}CO_2$ fixation by this system is not due to inhibition of carboxydismutase which would prevent entry of CO_2 into the cycle, but is primarily due to inhibition of triose phosphate dehydrogenase. The possibility of non-rate-limiting inhibition of some other enzymes acting between triose phosphate and ribulose diphosphate is not, however, ecxluded.

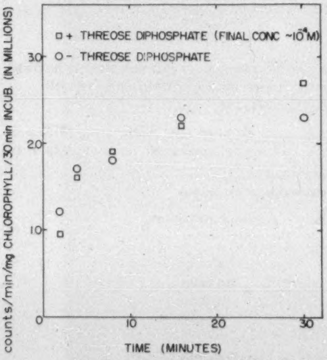

Fig. 2. The time course of $^{14}CO_2$ fixation by carboxydismutase in a sonically ruptured chloroplast system with and without threose diphosphate. Each reaction mixture contained 4.0 μM $NaH^{14}CO_3$ (100 μC), 0.2 μM RuDP and 1 ml of sonically ruptured chloroplasts in 10^{-3} M potassium phosphate (pH 7.4) containing 0.59 mg of chlorophyll. Mg^{++}, Mn^{++}, ADP, TPN, TPP, ascorbate and GSH concentrations are given in Table I. The reaction mixtures minus $NaH^{14}CO_3$ were preincubated for 1 min at room temperature after which the bicarbonate was added. The reaction mixtures were shaken in the dark for the prescribed time at 24° in air tight 35-ml vessels. $^{14}CO_2$ fixation by the same enzyme system minus RuDP in light was $21 \cdot 10^6$ counts/min/mg chlorophyll/30 min incubation without threose diphosphate and $7.4 \cdot 10^6$ counts/min/mg chlorophyll/30 min incubation with 10^{-4} M threose diphosphate.

The data plotted in Fig. 2 also show that the carboxydismutase rate observed during the first 2 min of the assay is sufficient to account for the rate of $^{14}CO_2$ fixation observed in the light sample.

TABLE III

THE INHIBITION OF PURIFIED CARBOXYDISMUTASE BY THREOSE DIPHOSPHATE

To each reaction mixture were added, in the order listed, the following: water or threose diphosphate solution to make 0.20 ml final volume; $NaH^{14}CO_3$, 1.35 μM; $MgCl_2$, 2 μM; Tris at pH 8.3, 15 μM; carboxydismutase, 23 μg; and RuDP, approx. 0.07 μM. The enzyme was preincubated with the inhibitor, bicarbonate and Mg^{++} at 25° for 2 min. At the end of this period, the incubation was started by adding RuDP. The reaction mixture was incubated for 10 min at 25°. The reaction was stopped with 50 μl 6 N HOAc. A 10-μl aliquot was plated and counted.

Threose diphosphate final conc (M)	*Counts/min/10 µl aliquot*	*Percent inhibition*
0	16,000	—
$8 \cdot 10^{-6}$	15,000	6
$8 \cdot 10^{-5}$	14,000	15
$8 \cdot 10^{-4}$	7,600	53

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REFERENCES

[1] J. A. Bassham and M. Calvin, *The Path of Carbon in Photosynthesis*, Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1957.

[2] O. Kandler, *Z. Naturforsch.*, 12b (1957) 271.

[3] E. Racker, V. Klybas and M. Schramm, *J. Biol. Chem.*, 234 (1959) 2510.

[4] A. Fluharty and C. Ballou, *J. Biol. Chem.*, 234 (1959) 2517.

[5] N. E. Tolbert, *The Photochemical Apparatus, Its Structure and Function*, Brookhaven Symposia in Biology, No. 11 (1958) 271.

[6] F. R. Whatley, M. B. Allen, L. L. Rosenberg, J. B. Capindale and D. I. Arnon, *Biochim. Biophys. Acta*, 20 (1956) 462.

[7] O. Holm-Hansen, N. G. Pon, K. Nishida, V. Moses and M. Calvin, *Physiol. Plantarum*, 12 (1959) 475.

[8] D. I. Arnon, *Plant Physiol.*, 24 (1949) 1.

[9] P. B. Sogo, N. G. Pon and M. Calvin, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 387.

[10] J. Mayaudon, A. A. Benson and M. Calvin, *Biochim. Biophys. Acta*, 23 (1957) 342.

[11] J. R. Quayle, R. C. Fuller, A. A. Benson and M. Calvin, *J. Am. Chem. Soc.*, 76 (1954) 3610.

[12] A. Weissbach, B. L. Horecker and J. Hurwitz, *J. Biol. Chem.*, 218 (1956) 795.

[13] W. B. Jakoby, D. O. Brummond and S. Ochoa, *J. Biol. Chem.*, 218 (1956) 811.

[14] M. Calvin and P. Massini, *Experientia*, 8 (1952) 445.

Biochim. Biophys. Acta, 42 (1960) 27–33

MACROMOLECULAR VARIATION IN THE CHROMATOPHORES OF THE PHOTOSYNTHETIC BACTERIUM *RHODOSPIRILLUM RUBRUM*

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(Received November 16th, 1959)

SUMMARY

Antisera were prepared in rabbits by injection of purified chromatophore preparations from *Rhodospirillum rubrum* grown anaerobically in the light. The antisera contained antibody which precipitated the pigmented particles from cell extracts, and could therefore be defined as chromatophore antibody.

By absorption of antisera with extracts of dark-grown cells devoid of pigment, two types of chromatophore antibody could be distinguished; one fraction of antibody reacted only with extracts of light-grown cells, the other fraction of antibody capable of precipitating pigmented particles reacted also with colorless antigens present in dark-grown cells. It was concluded therefore that chromatophore antigens are of two types, specific components formed as a consequence of photosynthetic growth, and antigens structurally related to chromatophores which are carried along in dark-grown cells.

Specific cell surface antigens formed during photosynthetic growth were detected by agglutination titrations of light-grown cells.

Photosynthetically grown cells placed in the dark for a short time alter their cellular contents such that chromatophore antibody no longer precipitates the pigmented component present in cell extracts. The evidence indicates, therefore, that the photosynthetic unit is capable of dissociation into fragments in the dark. This dissociation may help to explain induction and chromatic adaptation phenomena in photosynthesis.

INTRODUCTION

Most strains of photoheterotrophic bacteria can be grown either anaerobically in light or aerobically in the dark. In his classical studies on this group of microorganisms, VAN NIEL[1] pointed out that similar metabolic pathways were used by the organisms under both conditions. The cells can use oxygen in the dark as terminal electron acceptor, or can generate photooxidants as terminal electron acceptors for anaerobic growth in the light. Apart from the need for bacteriochlorophyll to provide photooxidants in cells growing anaerobically there are no known cellular alterations necessary for photosynthetic growth of these bacteria. Any cellular components formed exclusively during growth in the light could reasonably be considered essential parts of the photosynthetic apparatus.

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This report describes an attempt to detect, by immunochemical means, cellular constituents formed solely as a consequence of photosynthetic growth. Such unique antigens have been found. Analysis of the formation and macromolecular alteration in these structures appears to permit new insight into the general problem of metabolic induction phenomena in photosyntheses.

MATERIALS AND METHODS

Rhodospirillum rubrum was grown in the medium of COHEN-BAZIRE *et al.*[2] either in completely full 1-l reagent bottles in the light, or in 500-ml volumes of media in 2-l Erlenmeyer flasks rapidly shaken on a rotary shaker in the dark at 30°. All antigen preparations were from extracts of cells prepared in a Raytheon sonic oscillator. A purified "small particle" fraction of chromatophores was used for injection into rabbits to obtain antisera[3]. Sera were obtained from three rabbits after one month of a series of intravenous injections. The sera were pooled, heated to inactivate complement and used for this study. The general immunochemical methods used have been described[4].

RESULTS

When *Rhodospirillum rubrum* is cultured and subcultured aerobically in the dark for many generations, the cells can be depleted of bacteriochlorophyll and colored carotenoids until there is no spectroscopically detectable pigment present in cultures. Typical absorption spectra of extracts of cells grown in light and dark are shown in Fig. 1. The loss of pigment is phenomic; all of the bleached cells are capable of photosynthetic growth when returned to anaerobic conditions. When cells which have been grown in the light are disrupted, the entire photosynthetic pigment complex is found in "particles" 50 mμ in diameter, called "chromatophores"[5], which can be isolated by differential centrifugation. Cells grown aerobically in the dark do not release particles of this size on disruption.

Antisera were obtained toward purified chromatophore preparations derived from *R. rubrum* cells grown in the light. Extracts of both light-grown and of dark-grown cells reacted with the antisera, as seen in precipitin titration data in Fig. 2. Although the equivalence point in the two titrations was obtained with approximately the same amount of cell extract, the extract from light-grown cells precipitated more nitrogen on reaction with antiserum. Since nothing is known about the nitrogen content of the antigens precipitating in the two reactions one must ask whether the extract of light-grown cells is qualitatively different, apart from the obvious difference that the precipitate from its reaction contains the bacteriochlorophyll and carotenoids of the cell.

A large volume of antiserum was titrated to the equivalence point with an extract of dark-grown cells as indicated by the maximum in the curve in Fig. 2

(point a). The reaction mixture was allowed to stand 48 h in the cold and the precipitate was removed by centrifugation. The supernatant serum so obtained no longer reacted with extracts of dark-grown cells. It did, however, react with extracts derived from light-grown cells, as seen in the inset in Fig. 2. This supernatant serum, obtained by complete absorption with extracts of dark-grown cells, will be referred to as "specific antibody" since it contains antibodies which react with components present in light-grown, but not dark-grown cells.

As previously mentioned, the original antiserum precipitated bacteriochlorophyll from extracts of light-grown cells. Such a reaction can be defined as a chromatophore–antichromatophore reaction, because the antibody is capable of reacting with and specifically precipitating pigmented particles[4]. The chromatophore–antichromatophore reaction can be studied independently of other antigenically reacting species present in crude bacterial extracts because the precipitating antigen is a pigmented macromolecular complex which can be quantitatively measured by spectrophotometry of the immune precipitate. It is therefore possible to determine whether specific antibody and chromatophore antibody are the same, and if so, how much chromatophore antibody is specific only for light-grown cells.

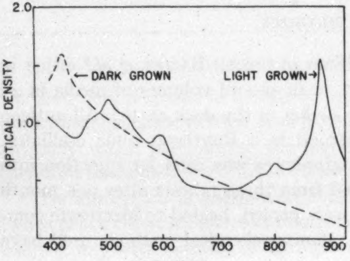

Fig. 1.

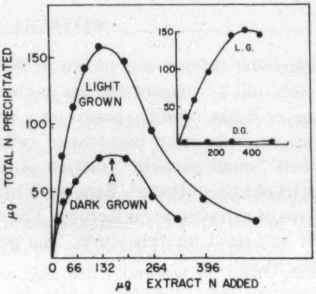

Fig. 2.

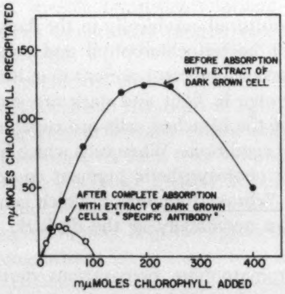

Fig. 3.

Fig. 1. Absorption spectra of sonic extracts of cells taken with Cary recording spectrophotometer. The extracts were adjusted to approximately equal protein concentration.

Fig. 2. Precipitin titrations of extracts and 0.1 ml antiparticle serum. Extract N plotted is acid-precipitable N only. The inset is a similar titration of serum equivalent to 0.5 ml after absorption at point "a" with extract of dark-grown cells.

Fig. 3. Reaction of pigmented particles from sonic extract with 0.5 ml antiserum and equivalent amount of antiserum after absorption at point "a" with extract of dark-grown cells.

Fig. 3 shows the results of a precipitin titration made with an extract of light-grown cells and the original antiserum, and a similar titration of the absorbed serum or "specific antibody". The data are adjusted to correct for dilution of the serum which occurs in the first reaction to prepare specific antibody. Prior reaction of the serum with an extract of dark-grown cells reduces, but does not completely eliminate the ability of the serum to precipitate pigment bearing particles. Approximately 20 % of the antibody capable of precipitating pigmented particles is formed to unique cellular components present only in light-grown cells. The largest fraction of chromatophore antibody reacts also with extracts of dark-grown cells. This antibody fraction will be referred to as "related antibody" since it reacts with components which although present on pigmented particles are also present in some form in dark-grown cells.

Previous work on immune reactions of pigmented particles from the photosynthetic anaerobe *Chromatium* has established that chromatophore antibody reacts with intact cells[4]. Data in Table I show that specific antibody can agglutinate intact cells of *R. rubrum* growing in the light. Such cells placed under aerobic growth conditions in dark lose the ability to be agglutinated however, and the reaction reappears when the culture is returned to anaerobiosis in the light. These data further indicate the cell surface nature of some of the specific components of the photosynthetic apparatus.

TABLE I

AGGLUTINATION OF INTACT *R. rubrum* CELLS BY "SPECIFIC ANTIBODY"

Specific antibody, 1:5 in reaction mixture (ml)	*$2 \cdot 10^9$ cells each type*		
	Light-grown cells	*Aerated-dark 7 h*	*Returned to light 13 h*
1.0	4 +*	+ —	4 +
0.8	4 +	+ —	4 +
0.7	3 +	—	3 +
0.6	2 +	—	2 +
0.5	2 +	—	2 +
0.4	2 +	—	2 +
0.3	+	—	—
0.2	+	—	—
0.1	—	—	—

* A reaction of 4 + indicates that all of the cells added were strongly agglutinated by visual estimate. The relative extent of agglutination was graded downward from this estimate by visual comparison. Total reaction volume 1.2 ml containing $2 \cdot 10^9$ cells and indicated amount of serum diluted in saline.

We have studied the ability of the types of chromatophore antibody to detect the presence of their respective antigens in cells growing in light and dark. The antigen is particulate, and consequently its titer may depend on its state of fragmentation during preparation of extracts by sonic rupture of the cells. Therefore, a series of control experiments was performed to test the reproducibility of the assay with cell extracts. The precipitin titrations were found to be reproducible on extracts provided an equal amount of cell breakage had been obtained. Data from a typical control experiment are given in Table II. Inquiry into the formation of chromatophore antigens, therefore, seemed possible.

It has been shown by COHEN-BAZIRE *et al.*[2] that aeration of photosynthesizing cells of *R. rubrum* causes an immediate cessation of chlorophyll synthesis but does not inhibit exponential growth of the cells. The data in Fig. 4 confirm the findings

TABLE II

TEST OF REPRODUCIBILITY OF PRECIPITIN REACTIONS ON SONIC EXTRACT

Values are given for the maximum in the precipitin titration curve. The shapes of the curves were identical with all extracts. The original antiserum, diluted 1 : 10, was used. Titrations were done on 1-ml aliquots of diluted serum.

	Precipitin titration of sonic extract	
Sonic oscillation (min)	*Bacteriochlorophyll precipitated (mμmoles)*	*Total N precipitated (μg)*
1	23.7	148
4	24.2	151
10	23.2	146

of Cohen-Bazire *et al.* and include serological analysis of cell extracts prepared from *R. rubrum* during growth in light and dark. A culture of *R. rubrum* growing exponentially under anaerobic conditions in the light was transferred to Erlenmeyer flasks, shaken vigorously on shaker for 10 h in the dark, and then returned to anaerobiosis in light. Growth and chlorophyll synthesis were measured spectrophotometrically, and aliquots of the culture were taken to prepare cell extracts for antigen titration. The growth curve of the culture and the relative amount of chlorophyll per cell are plotted in Fig. 4. Aeration resulted in an immediate inhibition of chlorophyll synthesis and consequent exponential decrease in the amount of chlorophyll per cell as growth continued aerobically. Returning the culture to anaerobiosis in light caused a resumption of photosynthetic growth and chlorophyll biosynthesis. No lag phase in growth was detectable in the culture during the conversion of cells from photosynthetic to aerobic growth or their return to photosynthesis, and no chlorophyll destruction took place in the cultures under any conditions. Precipitin titrations on extracts of these cells, using specific antibody, are also given in Fig. 4.

These data show that upon conversion of cells to aerobic growth in the dark, the chlorophyll in the cell becomes non-precipitable by specific antibody. On return to photosynthetic growth conditions, the ability of pigmented particles to react with specific antibody reappears. No chlorophyll destruction takes place during aerobic

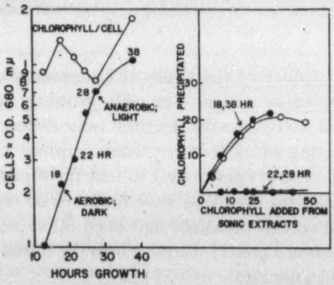

Fig. 4. Growth curve of *R. rubrum* culture initially growing exponentially in light. The bacteriochlorophyll content per cell was estimated from the ratio of O.D. at 880 and 680 mμ. In the precipitin titrations (inset) of cell extracts, standardized on chlorophyll content, specific antibody equivalent to 0.5 ml antiserum was used.

growth. The amount of chlorophyll added, in the form of crude bacterial extract, is the same for titration of all extracts. It is concluded, therefore, that photosynthetically grown cells of *R. rubrum* which have subsequently been allowed to grow for a short time aerobically in the dark alter their cellular contents in a manner which renders their chlorophyll non-precipitable by specific antibody.

The question arises whether related antibody, which can react with both pigmented particles and dark-grown cell extracts, reacts with the pigmented particles in the aerated cells. Data in Table III show that there is a large decrease in the ability of the original antiserum to precipitate pigmented particles from the extract from aerated cells. This decrease is greater than can be accounted for by loss of reaction by specific antibody alone. However, the presence of non-pigmented reactants in these cells is indicated by the amount of nitrogen in the precipitate from the aerated cell extract.

TABLE III

REACTION OF PIGMENTED PARTICLES WITH ORIGINAL ANTISERUM

The original antiserum was diluted 1:10 and 1-ml aliquots used for titration. Data are given for the maximum in the titration curves.

History of cells	*Precipitin titration of sonic extracts*	
	Bacteriochlorophyll precipitated (mμmoles)	*Total N precipitated (μg)*
Initial culture growing in light anaerobically	25.0	158
Placed in dark 4 h, aerobic	4.5	136
Returned to light for 10 h	26.4	156

Extracts prepared from photosynthetically grown cells of *R. rubrum* which had been washed and aerated in phosphate buffer did not lose their ability to precipitate pigmented particles on reaction with specific antibody. These data are given in Table IV. Therefore, growth of the cells is required to alter the photosynthetic apparatus.

We next attempt to analyze the nature of the loss of reactivity by the pigmented particles of *R. rubrum* during aerobic growth. It may be asked whether the unreactive particles are uniquely different from those originally present in photosynthesizing

TABLE IV

STABILITY OF PIGMENTED PARTICLES IN AERATED RESTING CELLS

Specific antibody equivalent to 0.5 ml original serum was used.

Initial cells, light grown		*Cells aerated 3 h, 0.05 M phosphate, pH 6.8*	
Precipitin reaction, bacteriochlorophyll (mμmoles)		*Precipitin titration, bacteriochlorophyll (mμmoles)*	
Added	*Precipitated*	*Added*	*Precipitated*
18	12	16	12
36	22	32	22
54	14	64	14
72	12	96	12

cells. By aeration of growing cells for short periods of time, a partial loss of reactivity of particles in the cell extract could be obtained. The extract from aerated cells was then titrated with specific antibody to the equivalence point and the new absorbed serum obtained was retitrated with an extract of photosynthetically grown cells. The data are given in Table V. It can be seen that absorption of specific antibody with the extract of aerated cells which had a reduced titer completely removed all antibody capable of precipitating pigmented particles. It appears, therefore, that there is no preferential destruction of some specific antigens on the macromolecules in aerated, photosynthetically grown cells, accounting for the loss of reactivity.

TABLE V

ABSORPTION OF SPECIFIC ANTIBODY BY EXTRACT FROM AERATED CELLS

	History of cells	*Chlorophyll precipitated at equivalence (mμmoles)*
a	Initial light grown	27
b	Aerated	18
c	Returned to light	30
d	Reaction of c after absorption with b at equivalence point	0

TABLE VI

PRECIPITATION OF MIXTURES OF EXTRACTS FROM PHOTOSYNTHETICALLY-GROWN AND AERATED CELLS

Specific antibody equal to 0.5 ml original antiserum used per tube. When antigen mixtures were added, the aerated cell extract was added first.

Bacteriochlorophyll added as sonic extract of (mμmoles)		*Bacteriochlorophyll precipitated (mμmoles)*
Light-grown cells	*Light-grown cells + aerobic growth**	
15	0	14
30	0	26
0	17	2
15	7	14
15	15	12
15	29	6

* The light-grown culture was placed under exponential aerobic growth conditions in the dark for 10 h.

It seemed likely that photosynthetically grown cells formed non-pigmented reactants on aeration which could prevent precipitation of any intact chromatophores by specific antibody. The presence of such "inhibitors" in aerated cells was demonstrated by studying precipitation of extracts which reacted to precipitate chlorophyll, alone and in the presence of extracts from aerated cells. Typical data are given in Table VI, which shows that the precipitation of pigmented particles from reactive extracts by specific antibody could be inhibited by extracts of pigmented cells grown aerobically. This suggests the presence, in the aerobically grown cells of components which, although non-pigmented, react with specific antibody.

Finally, more precise characterization of the macromolecular change accompanying the inability of chlorophyll to be precipitated from aerated cell extracts was attempted by simultaneously studying antibody, chlorophyll and ^{32}P precipitation from extracts prepared from pigmented cells before and after aerobic growth. ^{32}P labeled cells were obtained by growth of the cells in $^{32}PO_4$ containing media to label the chromatophore phospholipids, which constitute the majority of the phosphorus of the particle, and consequently, provide a measure of precipitating antigen independent of chlorophyll content. The antibody used was a preparation of specific antibody conjugated with dimethylaminonaphtalene sulfonyl chloride by the method of REDETZKI[6]. The relative amount of antibody in the precipitates was determined directly by measurement of the fluorescence of the washed specific precipitates in a Farrand fluorometer. The data are summarized in Table VII, and show that extracts of the aerated cells react with and precipitate specific antibody from the serum, although such extracts precipitate less chlorophyll on reaction. Furthermore, the composition of the specific precipitate formed by extracts of aerated cells is different from that precipitate formed by the contents of photosynthesizing cells sampled initially. There is less chlorophyll per unit phospholipid on the precipitating antigen from the aerated cells. These data are all consistent with a release of chlorophyll from its bearer antigen during aerobic growth of pigmented cells of *R. rubrum*.

TABLE VII

CHANGE IN COMPOSITION OF PIGMENTED ANTIGEN ON AEROBIC GROWTH OF *R. rubrum*

The data refer to different points on the respective precipitin curves.

Analysis of specific antibody precipitates from extracts							
Initial cells (light-grown)				*90 min aerobic growth*			
*Antibody**	*^{32}P (counts/min)*	*Bacteriochlorophyll (mμmoles)*	*Ratio***	*Antibody*	*^{32}P (counts/min)*	*Bacteriochlorophyll (mμmoles)*	*Ratio*
24	27,000	1.1	2.45	18	30,000	1.1	2.72
21	49,000	2.8	1.75	14	39,000	1.4	2.78
14	41,000	2.6	1.57	10	25,000	1.1	2.27
10	25,000	1.5	1.66	10	20,000	0.8	2.50

* Galvanometer readings indicating relative fluorescence of precipitates.
** ^{32}P, counts/min/bacteriochlorophyll × 10^4.

DISCUSSION

This investigation establishes the presence of unique antigenic components in *Rhodospirillum rubrum* which are formed as a specific consequence of photosynthetic growth. Although the chemical nature of these antigens is unknown at present, one of them appears to be a cell surface component, because specific antibody reacts with and agglutinates intact cells growing photosynthetically. A cell wall polysaccharide serologically related to the pigmented particles in the photosynthetic anaerobe, *Chromatium*, has been isolated[4]. It remains to be determined whether *R. rubrum* contains a similar surface antigen.

The great speed with which *R. rubrum* can alter its metabolism from photosynthetic to aerobic growth suggests that little change in intracellular architecture is required for conversion. We find from analysis of the immunochemical data that the changes occurring are principally characterized by the chlorophyll of the cell entering a non-precipitable form. The fact that extracts of aerated cells still react with and remove specific antibody from sera indicates that the unique chlorophyll-bearing antigens in these cells are neither masked nor destroyed; they are separated from the chlorophyll of the cell. Furthermore, the chlorophyll present in aerated cells is not precipitable by related antibody either, which indicates that an appreciable fraction of the macromolecules are disorganized, not just specific components formed in light.

Further data lending support for the view that aerobic growth leads to chlorophyll dissociation from the photosynthetic apparatus come from recent work on pigment mutants of *Rhodopseudomonas spheroides*. These cells lack unsaturated carotenoids and are killed by simultaneous exposure to light and oxygen[7]. Death of the cell results from a chlorophyll-mediated photooxidation of an unknown essential cellular constituent. DWORKIN has recently found that a short period of aerobic growth will protect the cell from photokilling[8]. This also suggests that during dark aerobic growth, the chlorophyll has entered a different form than its state in photosynthesizing cells. In the aerated mutant cell, chlorophyll may be no longer in contact with the essential cell component, and cannot cause death.

All of the data presented in this paper lead to the view that during aerobic growth, the chlorophyll of the cell leaves its bearer antigens. Whether this change involves alteration of the tertiary structure of the photosynthetic unit, or more extensive changes, is unknown. The dissociation does not appear to release free chlorophyll because there are no spectral changes of chlorophyll accompanying the reaction. However, we may ask how the separation might affect the organisms physiologically and whether this finding can be used to explain events following subsequent illumination of the cells. There is a long history of reports of "induction phenomena" in photosynthesizing cells placed aerobically in the dark for a period of time and subsequently returned to the light[9]. Theories attempting to explain the lag in photosynthesis and other physiological changes which occur in green cells returned to light have generally assumed that during the dark period the photosynthetic apparatus remained intact. The present results show that, in *R. rubrum* at least, such an assumption is untenable and immediately suggests an alternative explanation for metabolic induction periods; that it is simply the time required for chlorophyll to reorient itself in its site in the photosynthetic apparatus. Furthermore, some aspects of "chromatic adaptation" of green cells may be explained on this basis; cells grown in light which is not absorbed by chlorophyll may be expected by analogy with dark-grown cells to dissociate their photosynthetic unit and contain "inactive" chlorophyll of the type shown in this study to be separated from its bearer antigen(s) in the cell.

ACKNOWLEDGEMENTS

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REFERENCES

[1] C. B. van Niel, *Advances in Enzymology*, 1 (1941) 263.
[2] G. Cohen-Bazire, W. R. Sistrom and R. Y. Stanier, *J. Cellular Comp. Physiol.*, 49 (1957) 25.
[3] J. W. Newton and G. A. Newton, *Arch. Biochem. Biophys.*, 71 (1957) 250.
[4] J. W. Newton and L. Levine, *Arch. Biochem. Biophys.*, 83 (1959) 456.
[5] H. K. Schachman, A. B. Pardee and R. Y. Stanier, *Arch. Biochem. Biophys.*, 38 (1952) 245.
[6] H. M. Redetzki, *Proc. Soc. Exptl. Biol. Med.*, 98 (1958) 120.
[7] M. Griffiths, W. R. Sistrom, G. Cohen-Bazire and R. Y. Stanier, *Nature*, 176 (1955) 1211.
[8] M. Dworkin, *Bacteriol. Proc.*, (1959) 55.
[9] E. I. Rabinowitch, *Photosynthesis*, Interscience Press, New York, 1956.

HAEMOGLOBIN OF THE LAMPREY, *LAMPETRA FLUVIATILIS*

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SUMMARY

The haemoglobin of the brook lamprey (*Lampetra fluviatilis*) has been investigated. It has a molecular weight of 17,200 and an absorption spectrum in the visible and Soret regions very similar to human haemoglobin. The tryptophan fine-structure absorption band in the u.v. and amino-acid composition of lamprey and human haemoglobins are significantly different. Lamprey haemoglobin has one sulfhydryl group per mole of native and 1.9 group per mole of denatured protein. All 26 specimens examined showed one minor and two major components on electrophoresis. Lamprey haemoglobin is of special interest in providing a clue to the evolution of haemoglobin in the vertebrates.

INTRODUCTION

Cyclostomes are the most primitive living vertebrates. Together with the extinct ostracaderms, the most ancient fossil vertebrates, they form the super-class *Agnatha*, which has followed an independent evolution since earliest vertebrate origins. Cyclostomes are also the most primitive animals known to have haemoglobin enclosed in erythrocytes. Haemoglobin and myoglobin are two proteins which have been intensively studied both from the structural and the functional point of view. Investigations of haemoglobins in different species[1] show how the protein has been modified in the course of evolution to fulfil the physiological needs of a wide range of organisms. The haemoglobin of the lamprey is of special interest since it lies intermediate in structure and properties between myoglobins and haemoglobins of higher vertebrates, and so provides clues to the evolution of the whole group of proteins.

Although isolated studies of lamprey haemoglobin have been reported, there has been no systematic survey study of its properties. This paper reports the results of a series of investigations of the haemoglobin of the lamprey undertaken to that end.

MATERIAL AND METHODS OF STUDY

Twenty six adult specimens of the brook lamprey, *Lampetra fluviatilis*, and two specimens of the sea lamprey, *Petromyzon marinus*, were obtained commercially and bled into acid-citrate-dextrose solution. The washed red cells were lysed by addition of distilled water or exposure for 1 min to ultrasonic vibration from a magnetostriction transducer (25 kc/sec; 200 W) in an ice bath. Cell stroma was removed by centrifugation at 12,000 × g for 30 min. Electrophoresis was carried out in a starch block[2], using barbiturate buffer, pH 8.6; I 0.15, on 3 MM filter paper with the same buffer, and in agar using citrate buffer, pH 6.5 (see ref.[3]). Sedimentation was studied in phosphate buffer, pH 6.8, ionic strength 0.2, in a Spinco Model E ultracentrifuge at speeds of 16,200 rev./min for molecular weight determinations by the ARCHIBALD method[4,5], and at 59,780 rev./min for sedimentation measurements. Protein concentrations were 0.84 % and 0.42 %, respectively. The light filter was removed and exposure increased because of the strong absorption by the solutions; this made it impossible to obtain photographic records of the highest quality, but should not materially affect the accuracy of the results. Absorption spectra were measured in a Unicam S.P. 600 spectrophotometer, the trytophan fine-structure band being located by the moving-plate logarithmic cam spectrographic technique of HOLIDAY[6]. The concentration of haemoglobin solutions for titrations was measured spectrophotometrically, the millimolar extinction coefficient at 540 mμ being taken as 14.9 per atom of iron. The sulphydryl content of lamprey carboxyhaemoglobin, both native and denatured, was determined by amperometric titration with phenyl mercuric hydroxide using the dropping mercury indicator electrode as described by ALLISON AND CECIL[7] for adult human haemoglobin. Complete amino-acid determinations were made on 22- and 24-h hydrolysates, using the column chromatographic technique of MOORE AND STEIN as described elsewhere[8,9].

RESULTS

Molecular weight

SVEDBERG AND ERIKSON-QUESNEL[10] deduced from sedimentation and diffusion studies that the haemoglobin of the brook lamprey, *Lampetra* (= *Petromyzon*) *fluviatilis*, had a molecular weight of 19,100, whereas that of the hagfish, *Myxine glutinosa*, was 23,100. LENHERT, LOWE AND CARLSON[11], using ACHIBALD's method, reported a molecular weight of 23,600 for haemoglobin from *P. marinus*. Both groups obtained lower values from haem and iron analyses (17,250–18,600, assuming one haem per mole), and therefore concluded that there was some aggregation into dimers. We have repeated the molecular weight determination by ARCHIBALD's method[4,5] on haemoglobin purified by electrophoresis in starch blocks. The mean value, from five exposures taken between 30 and 60 min after reaching speed (16,200 rev./min) was 17,200, which is consistent with the observed sedimentation coefficient ($S_{20,w}$ = 1.80 Svedberg units). In the calculations $\overline{V}$ was taken as 0.731, the value calculated from the amino-acid composition[12–14]. If the value of 0.751 used by other workers is substituted, the molecular weight is a little higher (18,600). It was not possible to distinguish more than one reasonably symmetrical peak in the high velocity experiments, but the rather rapid broadening of the peak by diffusion would have made it difficult to carry out a detailed analysis of the curve for evidence of heterogeneity. Certainly there was no significant trend of molecular weight with time in the low-speed experiment, which would be expected if small aggregates were present in significant proportions.

Electrophoresis

The haemoglobin of the sea lamprey (*P. planeri*) has shown two major haemoglobin components on starch gel electrophoresis[14–16]; the larval form of the lamprey also has two components, both of which are stated to be different from those in adults. On the other hand the Pacific lamprey (*Entosphenus tridentatus*) appears to have a single haemoglobin component on filter paper electrophoresis[17].

All 26 specimens of *L. fluviatilis* haemoglobin submitted to filter paper electrophoresis at pH 8.6 were found to have two major components and one small minor component. The latter was well shown after benzidine staining. The same components were observed on starch block electrophoresis and electrophoresis in agar at pH 6.5 (Fig. 1). Components with similar mobilities were seen in two specimens of haemoglobin from the sea lamprey, *P. marinus*, submitted to filter paper electrophoresis. The possession of two major haemoglobin components would therefore appear to be the rule in all adult members of the genera *Lampetra* and *Petromyzon*, but not of *Entosphenus*. The isoelectric point of lamprey haemoglobin in phosphate buffer, ionic strength 0.05, is $pI = 5.6–5.7$, falling within the range of isoelectric points of invertebrate haemoglobins ($pI = 4.5–6.0$) as opposed to those of most higher vertebrates ($pI = 6.7–7.2$)[18,19].

Fig. 1. Two major components (1 and 2) and a minor component (3) of the haemoglobin of *Lampetra* after electrophoresis in agar at pH 6.5. The origin is marked 0.

Amino acid composition

The amino acid composition of lamprey haemoglobin, purified by starch block electrophoresis, is given in Table I. Amino-acid compositions of human myoglobin[20] and haemoglobin[21], and the haemoglobin of a teleost fish, *Thunnus thynnus*[22], are included for comparison. It will be noted that there are considerable differences in the proportions of several amino acids from human myoglobin and from the haemoglobins of higher vertebrates.

Attempts were made to determine the N-terminal amino acids of both lamprey haemoglobin components by the fluorodinitrobenzene method[23]. No N-terminal amino acids were detected by this technique, which has previously been applied successfully to the haemoglobins of several other species. This result does not exclude the possibility that N-terminal proline is present, but suggests that the N-terminal amino groups may be blocked, *e.g.* by acetylation or cyclization.

TABLE I

AMINO ACID COMPOSITION OF LAMPREY HAEMOGLOBIN, HUMAN MYOGLOBIN AND THUNNUS AND HUMAN HAEMOGLOBINS

Composition in grams of amino acid/100 g protein.

Amino acid	*Lamprey Hb*	*Human Mb*	*Thunnus Hb*	*Human Hb*
Aspartic acid	9.3	8.3	11.2	9.6
Glutamic acid	7.2	16.2	6.7	6.6
Glycine	2.4	6.1	4.3	4.3
Alanine	10.3	5.6	8.5	9.2
Valine	8.2	3.4	7.3	10.4
Leucine	4.3	8.9	11.7	14.0
Isoleucine	4.4	3.4	5.7	< 0.02
Serine	8.3	4.4	4.5	4.1
Threonine	4.4	2.9	4.4	5.1
Methionine	2.9	1.5	2.8	1.3
Proline	4.6	4.8	2.4	5.0
Phenylalanine	8.3	4.3	6.4	7.3
Tyrosine	4.3	1.0	4.6	3.1
Histidine	1.0	12.9	4.1	8.3
Lysine	10.4	22.3	8.6	9.3
Arginine	2.9	2.5	8.6	2.8

Sulfhydryl groups

The number of sulfhydryl groups in lamprey carboxyhaemoglobin, both native and denatured, was determined by amperometric titration with phenyl mercuric hydroxide. The buffers used were borate ($Na_2B_4O_7$, 0.02 *M*; KCl, 0.15 *M*) and ammonia (NH_4Cl, 1.0 *M*; NH_4OH, 1.0 *M*). The protein was denatured by the addition of sodium dodecyl sulphate (approx. 400 moles/mole of protein). The native protein was found to have 1.0 SH per molecule (mol. wt. 17,000) and the denatured protein 1.9 SH per molecule. The same titres were obtained in both borate and ammonia buffers. Titrations of both the native and denatured protein were carried out in the presence and absence of sodium sulfite (0 to 0.1 *M*). The fact that the titres were independent of the sulfite concentration is evidence that there are no disulfide bonds in the molecule.

Absorption spectrum

The absorption spectrum of lamprey carboxyhaemoglobin has been examined in detail from 250–650 mμ. In the range 320–650 mμ the spectrum appears to be identical with that of human adult carboxyhaemoglobin (α band at 574 mμ, β at 540 mμ γ at 418 mμ and δ at 343 mμ, with the same extinction ratios). However, differences were apparent at shorter wavelengths. The ratio of predominantly protein absorption at 290 mμ to haem absorption at 343 mμ was 1.16 as compared with 0.93 in normal adult human carboxyhaemoglobin. Moreover, the tryptophan fine-structure band was less prominent than in other haemoglobins and at an unusually long wavelength

(291.2 mμ or longer). The major haemoglobin components were examined as separated zones in agar and showed no perceptible differences in this respect.

DISCUSSION

Lamprey haemoglobin consists entirely of units with molecular weight 17,200 each containing a single haem and, presumably, a single polypeptide chain. As expected of a pigment in which very little or no haem–haem interaction is possible, the oxygen equilibrium curve is hyperbolic[24]. In this respect lamprey haemoglobin resembles myoglobin, but the oxygen affinity of the latter is much greater under comparable conditions. Moreover, in its absorption spectrum and other properties lamprey haemoglobin resembles the haemoglobins rather than the myoglobins of higher vertebrates. Thus, there is a pronounced Bohr effect, which may be related to the presence of a free sulfhydryl group in the molecule; in other vertebrate haemoglobins the magnitude of the Bohr effect is related to the number of free sulfhydryls[25]. Myoglobin, which has an extremely small Bohr effect, has no sulfhydryl groups. The haemoglobin of the Californian hagfish, *Poliostrema stouti*, belonging to another order of cyclostomes, is reported to be hyperbolic and to have no Bohr effect[26]; it would be of interest to determine whether any thiols are present in the molecule. The presence of a sulfhydryl group in lamprey haemoglobin also shows that haemoglobins containing these groups do not necessarily aggregate into dimers or tetramers.

The presence of two electrophoretically separable major components in all adult *Lampetra* and *Petromyzon* studied, and of two other components in larval *P. planeri*[15,16], suggests that the four haemoglobins are synthesized under the control of four distinct genes. The universal presence of multiple haemoglobins occurs in the horse, the fowl and many other vertebrates[1]; this situation is quite distinct genetically from the haemoglobin polymorphisms found in man, monkeys, cattle, sheep and goats, in which different members of the same species show different components. In man at least three haemoglobin genes are assumed to be present controlling the synthesis of α, β and γ polypeptide chains; foetal and normal adult haemoglobins are produced by combination of these chains into $\alpha_2\gamma_2$ and $\alpha_2\beta_2$ units, respectively (see ref. 27).

A tentative picture of the evolution of the haemoglobin molecule can be drawn from available evidence. It would appear likely that the first pigment of this type, capable of reversible combination with oxygen, arose through attachment of haem —present in all free-living organisms—to a protein of molecular weight about 17,000. It is possible that at one stage haemoglobin in blood cells and myoglobin in tissue cells were identical. In any case from very early times they followed independent evolutions, myoglobin remaining as a storage pigment with a high oxygen affinity and haemoglobin becoming progressively adapted to fulfil its main function of oxygen transport in different species. Two important events occurred during the adaptation of the latter[28]. One was the acquisition of a Bohr effect, which facilitates oxygen liberation in the tissues. This may have arisen through incorporation of cysteine into the molecule, and represents the stage of development found in lampreys today. The second event was the aggregation of units of molecular weight about 17,000 to give the haemoglobins of approx. 68,000 found in all higher vertebrates. This would have permitted haem–haem interaction during oxygenation, with the development of a sigmoid oxygen equilibrium curve which is nicely adapted to carry out oxygen transport under a wide range of conditions in higher organisms.

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REFERENCES

[1] W. B. Gratzer and A. C. Allison, *Biol. Rev.*, in the press.
[2] N. G. Kunkel, *Methods of Biochem. Anal.*, 1 (1954) 141.
[3] A. R. Robinson, M. Robson, A. P. Harrison and W. W. Zuelzer, *J. Lab. Clin. Med.*, 50 (1957) 745.
[4] W. J. Archibald, *J. Phys. Chem.*, 51 (1947) 1204.
[5] P. A. Charlwood, *Trans. Faraday Soc.*, 53 (1947) 874.
[6] E. A. Holiday, *J. Sci. Instrum.*, 14 (1937) 166.
[7] A. C. Allison and R. Cecil, *Biochem. J.*, 69 (1958) 27.
[8] S. Jacobs, *Res. Comun. XV Congr. Quim. Anal.*, Lisboa, 1956, p. 212.
[9] S. Jacobs, *Bull. Soc. chim. Belgrade*, in the press.
[10] T. Svedberg and I. B. Erikson-Quesnel, *J. Am. Chem. Soc.*, 56 (1934) 1700.
[11] P. G. Lenhert W. E. Lowe and F. D. Carlson, *Biol. Bull.*, 111 (1956) 293.
[12] E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids and Peptides*, Reinhold Publ., Corp., New York, N.Y., 1943, p. 370.
[13] T. L. McMeekin and K. Marshall, *Science*, 116 (1952) 142.
[14] P. A. Charlwood, *J. Am. Chem. Soc.*, 79 (1957) 776.
[15] M. Adinolfi, G. Chieffi and M. Siniscalco, *Nature*, 184 (1959) 1325.
[16] M. Adinolfi and G. Chieffi, *Nature*, 182 (1958) 730.
[17] [illegible]. R. Buhler and W. E. Shanks, *Science*, 129 (1959) 899.
[18] J. Roche and M. Fontaine, *Ann. inst. Oceanog. (Paris)*, 20 (1940) 77.
[19] K. O. Pedersen, in T. Svedberg, *The Ultracentrifuge*, Oxford, University press, 1940.
[20] A. Rossi-Fanelli, D. Cavallini and C. de Marco, *Biochim. Biophys. Acta*, 17 (1955) 81.
[21] W. H. Stein, H. G. Kunkel, R. D. Cole, D. H. Spackman and S. Moore, *Biochim. Biophys. Acta*, 24 (1957) 640.
[22] A. Rossi-Fanelli, C. de Marco and E. Antonini, *Nature*, 181 (1958) 1128.
[23] R. R. Porter and F. Sanger, *Biochem. J.*, 42 (1948) 287.
[24] G. Wald and A. Riggs, *J. Gen. Physiol.*, 35 (1951) 45.
[25] C. Manwell, *Biol. Bull.*, 115 (1958) 227.
[26] A. Riggs, *Nature*, 183 (1959) 1037.
[27] A. C. Allison, *Eugenics Quart.*, 6 (1959) 155.
[28] G. Wald in E. S. G. Barron, *Modern Trends in Physiology and Biochemistry*, Academic Press, N.Y., 1952, p. 337.

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THE FREE RADICAL NATURE OF AROMATIC HYDROXYLATION EFFECTED BY OXYGEN, ASCORBIC ACID AND FERROUS IONS

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SUMMARY

The hydroxylation of benzoic acid by oxygen in the presence of ascorbic acid, ferrous sulphate and ethylenediaminetetraacetic acid in a phosphate buffer is shown to give a mixture of mono- and di-hydroxybenzoic acids and the approximate proportions have been estimated. They are consistent only with a homolytic substitution. The presence of free radicals in the hydroxylation mixture has been confirmed by polymerisation experiments.

INTRODUCTION

In 1952 UDENFRIEND *et al.*[1] showed that a water soluble system obtained from rat liver could, in the presence of diphosphopyridine nucleotide and oxygen, catalyse the conversion of L-, but not D-phenylalanine to tyrosine. Subsequent studies showed that ascorbic acid increased the efficiency of the conversion of tyramine to hydroxytyramine by adrenal medula homogenates. UDENFRIEND *et al.*[2] then found that the adrenal tissue was unnecessary, and that the hydroxylation of tyramine, and a number of other compounds[3] could be effected at 37° by oxygen in the presence of ferrous sulphate, ascorbic acid, and ethylenediaminetetraacetic acid in phosphate buffer. This system has been used to hydroxylate the compounds listed in Table I, and it has been suggested that both compounds normal to living systems and unusual injested materials may be hydroxylated in this manner *in vivo*.

The mechanism of the hydroxylation has been subject to speculation. UDENFRIEND *et al.*, from their own results and those of WILLIAMS[4] suggested that the hydroxyl cation (HO^+) was involved as the attack always appeared to take place at positions of high electron density. They ruled out the possibility that a free radical reaction was involved on the grounds that substitution was not sufficiently random. Nevertheless on the same data, DALGLIESH[5] later suggested that free radicals were involved as they can attack electronegative sites. While this is correct it has been thoroughly established for some time[6] that free radicals also attack electropositive positions and that homolytic attack on aromatic systems generally takes place predominately at the *ortho* and *para* positions to a single substituent irrespective of whether this substituent is electron donating or attracting in nature.

TABLE I

COMPOUNDS HYDROXYLATED BY UDENFRIEND'S PROCEDURE

Substrate	*Products*	*Yield %*	*Identification*	*Reference*
Acetanilide	2-hydroxyacetanilide	12	DU	3
	4-hydroxyacetanilide	17	I	3
Aniline	4-aminophenol	9	D	3
Anthranilic acid	3-hydroxyanthranilic acid	?	C	5
	5-hydroxyanthranilic acid	?	C	5
Antipyrine	4-hydroxyantipyrine	43	I	3
Benzoic acid	3-hydroxybenzoic acid	?	?	4
Cyanobenzene	3-cyanophenol	?	?	4
1,3-dimethylxanthine	1,3-dimethyluric acid	13	DU	3
4-ethoxyacetanilide	4-hydroxyacetanilide	7	I	3
Hippuric acid	2-hydroxyhippuric acid	?	C	9
2-hydroxyphenylalanine	2,3-dihydroxyphenylalanine	?	C	5
	2,5-dihydroxyphenylalanine	?	C	5
	Catechol?	?	C	5
Indole-3-acetic acid	5-hydroxyindole-3-acetic acid	?	C	5
	7(?)-hydroxyindole-3-acetic acid	?	—	5
Kynurenine	3-hydroxykynurenine	?	C	5
	5-hydroxykynurenine	?	C	5
Nitrobenzene	2-nitrophenol	?	?	4
Phenylacetic acid	Not clearly indentified	—	C	5
Phenylalanine	Tyrosine	?	C	5
	2-hydroxyphenylalanine	?	C	5
Phenylethylamine	Tyramine?	?	C	5
Quinoline	3-hydroxyquinoline	15	I	3
Salicylic acid	Gentisic acid	55	I	3
Tryptamine	5-hydroxytryptamine	?	?	3
	7-hydroxytryptamine	?	?	3
Tryptophane	5-hydroxytryptophane	?	C	5
	7(?)-hydroxytryptophane	?	—	5
Tyramine	3,4-dihydroxyphenylethylamine	25	C	3
Tyrosine	3,4-dihydroxyphenylalanine	?	C	5
	Hydroquinone?	?	C	5

C, paper chromatographic comparison with authentic material; D, distribution comparison with authentic material; I, isolation and mixed m.p.; U, u.v. absorption comparison with authentic material.

The formation of hydroxyl cations (HO^+) at pH 6–7 is so unlikely that it can be discounted. In any case electrophilic substitution of the highly deactivated aromatic rings of nitrobenzene, cyanobenzene and benzoic acid is difficult to effect and only occurs under very vigorous anhydrous conditions. It is highly improbable therefore that Udenfriend's reaction proceeds through the intermediary of a hydroxyl cation. The other possibility, a reaction involving free radicals, is consistent with the positions of hydroxylation of all the compounds listed in Table I which possess activated aromatic rings; hydroxylation takes place at the *ortho* and *para* positions to the existing substituents.

The situation is not so clear with the compounds possessing deactivated rings. In the case of nitrobenzene 2-nitrophenol is formed, in excellent agreement with the observation that phenyl radicals also attack nitrobenzene predominately at position 2 and that hydroxylation also takes place at this position (Table IV); the isomer ratios obtained for these hydroxylations are only approximate. Free radical hydroxylation of quinoline would not be expected to occur only at position 3 as claimed because homolytic phenylation gives[7] 8-phenyl- (30 %) and 4-phenyl-quinoline (20 %) as major products along with 14 % of 3-phenylquinoline and some of all the other phenylquinolines. Both 2- and 4-hydroxyquinoline ("quinolones"), which are neither phenolic nor basic, would have been discarded in the isolation procedure used[3] in working up the quinoline oxidation and the formation of 8-hydroxyquinoline might also have been missed. Electrophilic substitution of quinoline normally takes place at positions 5 and 8 although some reactions of uncertain mechanism[8] can lead to substitution at position 3. Cyanobenzene and benzoic acid are attacked by phenyl and hydroxyl radicals predominately at positions 2 and 4 (Table IV). The suggestion (Table I) therefore that these compounds hydroxylate largely or solely at position 3 is the only one wholely inconsistent with the postulate of a free radical hydroxylation mechanism and a reinvestigation of the benzoic acid hydroxylation has been carried out. It has been shown that 3-hydroxybenzoic acid is in fact a minor product of the reaction and that the isomer ratio of the hydroxybenzoic acids formed is consistent only with a free radical substitution.

METHODS

The u.v. absorption spectra were measured on a Carey recording spectrophotometer.

The chromatograms were all done on Whatman No. 1 paper in descending isopropanol–ammonia (d 0.880)–water (8:1:1, v/v, solvent A), or ascending[9] in the organic phase of benzene–propionic acid–water (2:2:1, v/v, solvent B) unless otherwise specified. They were examined under a "Hanovia" u.v. lamp emitting 90 % of its u.v. radiation at 2537 Å, and sprayed with diazotised 4-nitroaniline[10] (DNA), diazotised sulphanilic acid (DSA), or ammoniacal silver nitrate.

Qualitative hydroxylation experiments

A phosphate buffer (250 ml) containing disodium hydrogen phosphate (0.947 g) and potassium dihydrogen phosphate (1.36 g) was shaken with ethylenediaminetetraacetic acid (1.0 g) until saturated, and after filtration ferrous sulphate (0.20 g), ascorbic acid (3.0 g) and the substrate (0.3 g) were added. The pH was then determined, and adjusted where desired and the mixture was shaken under oxygen (2 lbs./in^2) in a 500-ml flask for the appropriate time at room temperature. The solution was then strongly acidified (H_2SO_4), saturated with ammonium sulphate and extracted with ether (4 × 100 ml). The ether extracts were combined, evaporated, and chromatographed alongside authentic samples on the same papers.

Estimation of the isomer ratios in the hydroxylation of the benzoic acids

1. Benzoic acid or its 2- or 3-hydroxy derivative were hydroxylated at pH 3.1 (1 h). The ether extracts were evaporated to dryness and the residues taken up in methanol (0.5 ml). Known volumes (approx. 0.01 ml) of these solutions were chromatographed in descending isopropanol–ammonia–water alongside standard amounts of authentic hydroxybenzoic acids on the same paper. The spots were examined as regards u.v. absorption, colours were developed with DSA and ammoniacal silver nitrate, and concentrations of the standard substances were found which accurately represented the spots obtained from the hydroxylation mixtures. The final chromatograms were always done in duplicate, and the results obtained for the same compound using the different detection techniques agreed.

2. Benzoic acid was hydroxylated at pH 3.1, and 0.04 of the product was applied as a stripe (15 cm) to a Whatman 3 mm paper, and developed in isopropanol–ammonia–water until the solvent had travelled 40 cm. The bands consisting of 2-, 3-, and 4-hydroxybenzoic acids, and a band which contained benzoic and 2,5-dihydroxybenzoic acids, were located under u.v. light and by samples of the acids placed as "markers" at the edge of the chromatographic sheet. These bands were cut out, and separately eluted with methanol. The eluates were adjusted to suitable concentrations, acidified with one drop of concentrated hydrochloric acid, and then their u.v. absorption spectra were measured. Blank experiments were done with the chromatography paper only, and the recoveries of all the acids were determined by subjecting known mixtures in the phosphate buffer, at approximately the concentrations formed in the hydroxylation mixture, to the full isolation procedure. Corrections, due to impurities in the paper were comparatively large but the results were in agreement with those obtained by method (1).

Polymerisation experiments

Benzoic acid was hydroxylated (21 h) as above at pH 3.1 and 6.6 in the presence of methyl methacrylate (10 ml) and at the same time otherwise similar runs were made at the same pH's, in the absence of ascorbic acid. The reaction mixtures were acidified, extracted with chloroform, and the extracts washed with aqueous potassium bicarbonate, water and dried. The chloroform solution was then either evaporated to dryness (100° at 10 mm) to remove solvent and monomer or concentrated and diluted with methanol when polymerised methyl methacrylate was precipitated. The potassium bicarbonate washings were acidified, saturated with ammonium sulphate and extracted with ether, and the concentrated extracts chromatographed. In the absence of ascorbic acid no hydroxylation of the benzoic acid and no detectable polymerisation took place. In the presence of ascorbic acid 2-, 3- and 4-mono-, and 2,5-dihydroxybenzoic acids were formed along with poly-methyl methacrylate (approx. 0.5 g). The hydroxylation mixture, without substrate, also polymerised the ester under the above conditions (up to 4.0 g of polymer) but no detectable polymerisation occurred in the absence of ascorbic acid.

RESULTS AND DISCUSSION

The results obtained in the qualitative hydroxylations are listed in Table II. Hydroxylation proceeded much more readily at pH 6.6, than at pH 3.1, as under these conditions the formation of 3,4-dihydroxybenzoic acid from benzoic acid and of 2,4-dihydroxybenzoic acid from 4-hydroxybenzoic acid, could be detected after a reaction time of 1 h. A small number of unidentified materials, not listed, were detected after 21 h reaction in most instances, and especially from experiments carried out at pH 6.6.

It was not easy to detect 2,3-dihydroxybenzoic acid in the presence of 3-hydroxybenzoic acid by chromatography in the ammonia solvent, except by its fluorescence, as the materials travel together. Light spraying with the DSA reagent did however reveal the dihydroxy acid as a transient pink before being masked by the yellow colour due to 3-hydroxybenzoic acid; presumably 2,3-dihydroxybenzoic acid couples preferentially. The R_F's of the 2,3- and 2,5-benzoic acids formed from benzoic acid are somewhat lower than the authentic materials in the ammonia solvent because of

TABLE II

THE HYDROXYLATION PRODUCTS OBTAINED AFTER 1 H AT pH 3.1 AND IDENTIFICATION DATA

A, absorbs; b, bright; B, blue; Bk, black; Bn, brown; G, grey-brown; N, nothing observable; O, orange-brown; p, pale; P, pink; R, red; W, white.

Substrate	*Positions of hydroxyl groups in reaction products*	*R_F's (× 100) of products and (authentic materials) in solvent*		*Colours observed with*			
		A	*B*	*UV*	*DSA*	*DNA*	*Ag^+/NH_3*
Benzoic acid	2-	78 (80)	86 (85)	B	pY	R	N
	3-	54 (55)	52 (51)	N	Y	P	N
	4-	44 (41)	56* (53)	A	Y	R	N
	2,3-**	52 (57)	41 (41)	bB	Pi	Bn	Bk
	2,5-	61 (68)	27 (26)	bB	W	pBn	Bk
	3,4-***	12 (13)	15 (16)	N	pP	Bn	Bk
2-hydroxybenzoic acid	2,3-	51 (57)	42 (41)	bB	pP	Bn	Bk
	2,4-	35 (35)	Not resolved	B	Bn	O	G
	2,5-	64 (65)	25 (26)	bB	W	Bn	Bk
3-hydroxybenzoic acid	2,3-	57 (60)	44 (41)	bB	pP	Bn	Bk
	2,5-	68 (68)	27 (26)	bB	W	pBn	Bk
	3,4-	14 (13)	15 (16)	N	pP	Bn	Bk
4-hydroxybenzoic acid	2,4-***	35 (32)	33 (34)	B	Bn	O	G
	3,4-	14 (12)	18 (16)	N	pP	Bn	Bk

* Detected by its u.v. absorption in this solvent as 3-hydroxybenzoic acid moves to almost the same position.

** First detected after 4-h reaction.

*** First detected after 21-h reaction.

retardation by the large amount of benzoic acid present. A small quantity of a compound (R_F approx. 0.9 in solvent A) which possessed a similar u.v. fluorescence to that of salicylic acid, was invariably formed during the hydroxylation of salicylic acid. It gave no colour with DSA, and was not identical with a specimen of 2,6-dihydroxybenzoic acid (m.p. 170°) prepared from an impure (m.p. $< 110°$) commercial specimen (Lights) by paper chromatography. No 3,5-dihydroxybenzoic acid could be detected in the products from 3-dihydroxybenzoic acid.

The fact that UDENFRIEND's hydroxylation mixture initiates the polymerisation of methyl methacrylate is consistent with a free radical mechanism. This agrees with the observation[11], which we have confirmed, that benzoic acid does not hydroxylate in aqueous ethanol as the ethanol is preferentially oxidised to acetaldehyde. The fact that DALGLIESH[5] hydroxylated a number of compounds in solutions containing a little ethanol is of no consequence as this solvent would be rapidly removed by the air stream bubbled through his apparatus.

Table III shows the results obtained from the isomer ratio experiments. These agree well with a homolytic mechanism and are quite incompatible with an electrophilic attack on the ring.

No 3,4-dihydroxybenzoic acid could be detected in the products from benzoic acid after hydroxylation for 1 h. This suggests that the 2,5-dihydroxybenzoic acid is formed by the further hydroxylation of 2-hydroxy- but not of 3-hydroxy-benzoic acid, and on this premise the ratios in Table IV have been calculated. If the 2,5-dihydroxybenzoic acid were formed by the hydroxylation of salicylic acid a corresponding amount of 2,3-dihydroxybenzoic acid should be formed. However in spite of

TABLE III

ISOMER RATIOS OBTAINED IN THE HYDROXYLATIONS AND DETERMINED CHROMATOGRAPHICALLY

Substrate	*Approximate % conversion*	*Positions of hydroxyl groups in products*							
		2-	*3-*	*4-*	*2,3-*	*2,4-*	*2,5-*	*2,6-*	*3,4-*
Benzoic acid	1	5:	1:	2:	—	—	1 ~ 2	—	—
2-hydroxybenzoic acid	20	—	—	—	7:	Trace	5	—	—
3-hydroxybenzoic acid	6	—	—	—	5:	—	4:	—	8

TABLE IV

SOME ISOMER RATIOS OBSERVED IN HOMOLYTIC SUBSTITUTIONS

Substrate	*Attacking radical*	*Source of radical*	*Ratio of isomers formed in substitution at positions*					*References*
			2		*3*		*4*	
Benzoic acid	?	Ascorbic acid and Fe^{++}	6 ~ 7	:	1	:	2	This work
Benzoic acid	C_6H_5	Benzoyl peroxide	59	:	15	:	26	12
Benzoic acid	HO	H_2O and X-rays	5	:	2	:	10	13
Benzoic acid	HO	U.V. and Fe^{++}	Approx. 2	:	Approx. 2	:	Approx. 1	14
Benzoic acid	HO	U.V. and H_2O_2	10	:	5	:	5*	15
Nitrobenzene	HO	H_2O_2 and Fe^{++}	2.5–3	:	2–2.5	:	5–5.5	16
Nitrobenzene	HO	H_2O and X-rays	3.5	:	3.0	:	3.5	17
Nitrobenzene	C_6H_5	Benzoyl peroxide	6.2	:	1.0	:	2.8	6
Cyanobenzene	C_6H_5	Benzoyl peroxide	6	:	1	:	3	6

* 2,5- (: 2) and 3,4-dihydroxybenzoic acid (: 8) also formed.

intensive efforts no 2,3-dihydroxybenzoic acid could be detected on chromatograms of the products from several 1 h hydroxylations at pH 3.1; the 2,5-acid showed up very clearly on these chromatograms. One explanation is that the initial radical formed in the oxidation of benzoic acid is converted to 2,5-dihydroxybenzoic acid without the intermediary of 2-hydroxy-benzoic acid. It is also possible that other compounds on the chromatograms interfered with the detection. The DSA spray and the u.v. examination are about 10 times less sensitive for 2,3-dihydroxybenzoic acid than for its 2,5-isomer, but nervertheless the expected amount if, present, should have been detected readily.

The isomer ratio observed for the hydroxylation of benzoic acid in UDENFRIEND's mixture is in remarkable accord with that reported for the phenylation of benzoic acid. This latter value (Table IV) has been calculated from infrared absorption spectrum measurements and is comparatively very accurate. These results are not in such good agreement with the other, very approximate, hydroxylation data, except in so far as 2- and 4-substitution exceeds that at the 3-position.

The identity of the radical which actually attacks the substrate is a subject for speculation and further study. It must be produced in some way from the ascorbic acid, it need not be the hydroxyl radical, and the observation[2] that hydroxylation occurs after the destruction of all the ascorbic acid in the mixture is of particular relevance.

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REFERENCES

[1] S. Udenfriend and J. R. Cooper, *J. Biol. Chem.*, 194 (1952) 203.
[2] S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *J. Biol. Chem.*, 208 (1954) 731.
[3] B. B. Brodie, J. Axelrod, P. A. Shore and S. Udenfriend, *J. Biol. Chem.*, 208 (1954) 741.
[4] R. T. Williams, personal communication referred to in ref. 3.
[5] C. E. Dalgliesh, *Arch. Biochem. Biophys.*, 58 (1953) 214.
[6] D. H. Hey, in W. A. Waters, *Vistas in Free Radical Chemistry*, Pergamon Press, 1959; D. R. Augood and G. H. Williams, *Chem. Revs.*, 57 (1957) 123.
[7] K. H. Pausacker, *Australian J. Chem.*, 11 (1958) 200.
[8] M. J. S. Dewar and P. M. Maitlis, *J. Chem. Soc.*, (1957) 2521 and earlier papers.
[9] M. D. Armstrong, K. N. F. Shaw and P. E. Wall, *J. Biol. Chem.*, 218 (1956) 293.
[10] R. M. Acheson, Rachel M. Paul and R. V. Tomlinson, *Can. J. Biochem. and Physiol.*, 36 (1958) 295.
[11] P. Smith, private communication.
[12] R. C. Danneley and E. C. Gregg, *J. Am. Chem. Soc.*, 76 (1954) 2997.
[13] H. Loebl, G. Stein and J. Weiss, *J. Chem. Soc.*, (1951) 405.
[14] H. G. C. Bates, M. G. Evans and N. Uri, *Nature*, 166 (1950) 869; *J. Am. Chem. Soc.*, 75 (1953) 2754.
[15] E. Boyland and P. Sims, *J. Chem. Soc.*, (1953) 2966.
[16] H. Loebl, G. Stein and J. Weiss, *J. Chem. Soc.*, (1949) 2074.
[17] H. Loebl, G. Stein and J. Weiss, *J. Chem. Soc.*, (1950) 2704.

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CYTOCHROME *C* OXIDASE: THE EFFECTS OF LIPIDES AND SURFACE ACTIVE AGENTS ON ENZYMIC ACTIVITY

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SUMMARY

Cytochrome *c* oxidase prepared by fractionation of DPNH oxidase loses up to 90 % of its activity when suspended in 5 % sucrose. This loss of activity can be restored by surface active agents, deoxycholate and unsaturated lysolecithin. When frozen cytochrome *c* oxidase is thawed it separates into three layers which have different original activities, but which can be reactivated to the same level by these compounds. Similarly particles can be separated by centrifugation which have different initial activities, but which can be stimulated by lysolecithin to the same final activity.

Extraction of cytochrome *c* oxidase with acetone or mixtures of acetone and ethanol results in removal of some phosphorus and marked diminution of activity. This inactivation can be reversed to some degree by a number of phospholipides in the presence of deoxycholate. Much better reactivation is obtained with yeast lysolecithin. In no case is the degree of reactivation greater than the percentage of original phospholipide remaining in the preparations of enzyme.

INTRODUCTION

In recent studies HATEFI[1] has shown that cytochrome *c* oxidase, prepared by treating mitochondria with *tert.*-amyl alcohol, is stimulated by a lipoprotein isolated from mitochondria. Small increases in activity were observed when the system was supplemented with crude preparations of individual phospholipides. GREENLEES AND WAINIO[2], using cytochrome oxidase isolated from a KEILIN AND HARTREE preparation, showed that a number of crude phospholipides were capable of activating the enzyme. Earlier WITTER[3] had demonstrated that treatment of mitochondria with lysolecithin increased the cytochrome *c* oxidase activity and at the same time "uncoupled" oxidative phosphorylation.

The present report describes the effects of a variety of lipides and surface active agents on the enzymic activity of cytochrome *c* oxidase. The effects of these agents on acetone extracted cytochrome *c* oxidase is also presented.

EXPERIMENTAL

Cytochrome *c* oxidase was prepared as described previously[4] from reduced diphosphopyridine nucleotide oxidase (DPNH oxidase). In 30 % sucrose the most active preparations catalyze the oxidation of approx. 15 to 20 μmoles of reduced cytochrome *c*/mg protein/min. Phosphorus determinations were performed by the method of FISKE AND SUBBAROW[5]. Protein was determined by the biuret technique as described previously[6]. Ferrocytochrome *c* was prepared from Sigma Chemical Company cytochrome *c* (Type III) by reduction with sodium dithionite. Excess dithionite was removed by aeration. Ferrocytochrome *c* oxidase activities were determined spectrophotometrically as described previously[7]. Yeast lecithin, yeast lysolecithin, and derivatives of yeast lecithin were prepared by HANAHAN AND JAYKO[8]. The phospholipides from DPNH oxidase were prepared by HANAHAN using an unpublished chromatographic technique. Tweens No. 20, 40, 60, and 81 were obtained from the Atlas Powder Company and tris(hydroxymethyl)aminoethane from Sigma Chemical Company.

Cytochrome *c* oxidase was lyophilized and then extracted with acetone as follows: 100 mg of the powdered enzyme were placed in a 50-ml centrifuge tube and 40 ml of cold acetone was added. The mixture was shaken repeatedly for 1 to 4 h and then centrifuged. This was repeated 4 to 6 times so that extraction time varied from 4 to 24 h. The entire procedure was performed at 4°. After the last extraction the suspension was filtered quickly in a Buchner funnel and the residue was taken up immediately in 0.02 *M* Tris buffer pH 7.5. In order to remove additional phospholipide, concentrations of 5, 10, 20, and 40 % ethanol were added to the acetone in other experiments.

In the reactivation studies the compound to be tested (dissolved in chloroform) was placed in a tapered centrifuge tube. The chloroform was evaporated and the residue suspended in 0.1 ml of 0.02 *M* phosphate buffer pH 7.5. To this mixture was added 0.1 ml of enzyme (5 mg protein/ml). Necessary dilutions were made with 0.02 *M* phosphate buffer pH 7.5 and reaction rates were carried out.

RESULTS

Table I shows the maximal stimulating effects of yeast lecithin, deoxycholate, yeast lecithin plus deoxycholate, and yeast lysolecithin on preparations of cytochrome

TABLE I

THE EFFECTS OF VARIOUS AGENTS ON THE ACTIVITY OF PREPARATIONS OF CYTOCHROME *c* OXIDASE

All activities expressed as μmole cytochrome *c* oxidized/mg protein/min.

Preparation	*Initial activity*	*Substance used in stimulating activity*			
		Yeast lecithin	*Deoxycholate*	*Yeast lecithin plus deoxycholate*	*Yeast lysolecithin*
1	1.2	2.3	7.0	7.0	
2	1.0	2.1	6.3	10.8	
3	0.6	1.0	3.9	3.6	
4	1.2		3.8		10.8
5	1.0		1.3		11.2
6	1.0		4.3		10.7

c oxidase. The enzyme preparations were stored in 5% sucrose and had low initial activities. Yeast lecithin increased the activity to nearly twice the initial level. Deoxycholate and deoxycholate plus lecithin stimulated the enzymic activity 5 to 10 times. Combinations of yeast lecithin and deoxycholate were not consistently superior to deoxycholate alone. The amounts of deoxycholate and/or yeast lecithin required for maximal stimulation varied from preparation to preparation. The most remarkable and consistent stimulation of the enzyme was obtained by adding yeast lysolecithin. Again the amount necessary varied with the preparation. When the turbid enzyme suspension was added to the lysolecithin or deoxycholate the suspension clarified. Maximum stimulation of enzymic activity was present when the suspension cleared. Because deoxycholate and lysolecithin had similar effects on solubility of suspensions of cytochrome *c* oxidase and on the catalytic activity of the enzyme, the influence of lysolecithin on DPNH oxidase was tested. Lysolecithin "opened" DPNH oxidase in

TABLE II

ACTIVITIES OF LAYERS OF CYTOCHROME *c* OXIDASE RESULTING FROM FREEZING AND THAWING OR CENTRIFUGING

All activities expressed as μmoles cytochrome *c* oxidized/mg protein/min.

Layer	*Initial activity*	*Activity after addition of yeast lysolecithin*
Freezing and thawing		
Top		
Hazy olive-green	4.0	14.2
Middle		
Hazy green-brown	2.6	14.5
Bottom		
Clear green-brown	6.7	14.0
Centrifugation		
1. Supernatant	6.0	12.4
Residue	1.5	11.0
2. Supernatant	4.0	10.0
Residue	1.0	11.4

the presence of histidine buffer in the same manner as has previously been reported for deoxycholate[7].

When frozen preparations of cytochrome oxidase were thawed, 3 distinct layers were observed. Table II shows the activities of these layers before and after stimulation with lysolecithin. The enzyme preparations used in the study were stored frozen in 0.02 *M* Tris buffer and did not show the marked diminution of activity that occurs with preparations stored in 5 % sucrose. The top layer was olive green in color and cloudy in appearance; the middle layer a cloudy greenish brown and the bottom layer greenish brown and clear. Although the various layers had different initial activities, the final stimulated activities were the same. Also shown in Table II, when cytochrome *c* oxidase suspended in Tris buffer is centrifuged at 124,000 × *g* for 10 min, particles are separated which have a lower specific activity than the supernatant enzyme. On stimulation with lysolecithin both fractions have the same catalytic activity.

TABLE III

THE EFFECTS OF A VARIETY OF COMPOUNDS ON THE ACTIVITY OF ACETONE-TREATED CYTOCHROME *c* OXIDASE

All activities expressed as μmoles cytochrome *c* oxidized/mg protein/min.

	Acetone-treated
Activity prior to acetone treatment	12.0
Activating compound	
None	0.2
Yeast lysolecithin	4.0
Yeast lecithin	0.4
Deoxycholate	0.4
Deoxycholate plus yeast lecithin	0.7
Ubiquinone	0.2
α-tocopherol	0.3
Digitonin	0.2
Saponin	0.2
Phospholipid from DPNH oxidase	0.4

Table III shows the effects of a number of substances on the reactivation of acetone treated cytochrome *c* oxidase. Acetone extraction reduced the activity of cytochrome oxidase to approximately 2 % of the original value. Subsequent treatment with deoxycholate, yeast lecithin, a variety of lipides from DPNH oxidase and combinations of lecithin and deoxycholate produced a 2- to 3-fold increase in the activity of the acetone-treated preparations. Upon exposure of the cytochrome *c* oxidase to yeast lysolecithin the activity increased to one-third of the original value. Ubiquinone, α-tocopherol, digitonin, and saponin had no appreciable effect. A saturated lysolecithin prepared from egg lecithin and lysolecithinic acid were approximately one-half as effective as the unsaturated yeast lysolecithin. Choline and glycerylphosphorylcholine were ineffective.

Table IV shows the concentrations of total phosphorus in four acetone extracted preparations of cytochrome oxidase and the maximally stimulated activities of the preparations. Neither the duration of extraction nor the number of changes of acetone were directly related to the amount of phosphorus remaining in the preparations.

TABLE IV

THE ACTIVITY OF ACETONE-TREATED CYTOCHROME *c* OXIDASE AND TOTAL ENZYME PHOSPHORUS

All activities expressed as μmoles cytochrome *c* oxidized/mg protein/min.

Preparation	*Stimulated* activity before extraction*	*Total P before extraction (mg/100 mg protein)*	*Activity after extraction*	*Total P after extraction (mg/100 mg protein)*	*Stimulated* activity after extraction*	*% of original P remaining after extraction*	*% of original stimulated activity remaining*
1	12.5	0.67	0.31	0.40	4.3	60	34
2	12.5	0.67	0.22	0.24	3.1	36	25
3	14.1	0.65	3.0	0.54	5.0	83	36
4	11.5	0.75	0.53	0.21	1.6	28	14

* Maximum activity obtained with lysolecithin added to enzyme.

TABLE V

A COMPARISON OF THE ACTIVITIES AND LIPIDE PHOSPHORUS OF TWO PREPARATIONS OF CYTOCHROME *c* OXIDASE EXTRACTED WITH ACETONE AND MIXTURES OF ACETONE AND ETHANOL

All activities represent the maximum activity obtained with lysolecithin stimulation and are expressed as μmoles cytochrome *c* oxidized/mg protein/min.

Enzyme	*Maximum stimulated activity*	*Lipide-extractable P (mg/100 mg protein)*	*% P remaining*	*% activity remaining*
Before extraction	7.3	0.28	100	100
Extracted with:				
acetone	5.1	0.20	71	69
acetone + 5% ethanol	1.2	0.04	14	16
acetone + 10% ethanol	0.7	0.02	7	10
acetone + 20% ethanol	0.05	0.012	3.6	0.7
acetone + 40% ethanol	0.03	0.004	1.4	0.4
Before extraction	11.2	0.28	100	100
Extracted with:				
acetone	4.0	0.16	56	37
acetone + 5% ethanol	1.2	0.12	42	11
acetone + 10% ethanol	0.4	0.01	5	3.7
acetone + 20% ethanol	0.04	0.006	2	0.4
acetone + 40% ethanol	0.005	0.001	0.3	0.05

The percentage decrease in activity after acetone extraction was greater in all instances than the percentage of total phosphorus removed. In no case was the percentage of original activity restored equal to the percentage of original total phosphorus remaining.

Table V shows a comparison of the lipide phosphorus to maximal recovered activity. Here the percentages of lipide phosphorus and percentages of enzymic activity remaining after extraction are parallel and suggest the possibility of a relationship between enzymic activity and the presence of phospholipides in the enzyme preparations.

DISCUSSION

It has been shown that two surface active agents, deoxycholate and unsaturated (yeast) lysolecithin, will stimulate cytochrome *c* oxidase. Associated with the stimulation is a physical change, namely, solubilization, marked by clearing of the enzyme solution. This would suggest that possibly the stimulation is related to the size or configuration of the enzyme particles. Support for such an explanation is given by the results of experiments which show first, that different layers of frozen and thawed enzyme have different original activities but can be stimulated to the same level by lysolecithin, and second, that enzyme fractions which differ in centrifugal properties and activities are reactivated to the same level.

Cytochrome *c* oxidase treated with acetone and alcohol loses most of its activity and some of its phospholipide. Reactivation with the most effective agent, yeast lysolecithin, does not bring the activity of the enzyme up to the level of the phospholipide remaining in the preparations. It would appear that such stimulation does not reactivate the enzyme fraction from which phospholipide has been removed but makes the remaining intact enzyme more available for catalysis.

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REFERENCES

[1] H. HATEFI, *Biochim. Biophys. Acta*, 30 (1958) 648.
[2] J. GREENLEES AND W. W. WAINIO, *J. Biol. Chem.*, 234 (1959) 658.
[3] R. F. WITTER, A. MORRISON AND G. R. SHEPARDSON, *Biochim. Biophys. Acta*, 26 (1957) 120.
[4] B. MACKLER AND N. PENN, *Biochim. Biophys. Acta*, 24 (1957) 294.
[5] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[6] A. G. GORNALL, C. J. BARDWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
[7] B. MACKLER AND D. E. GREEN, *Biochim. Biophys. Acta*, 21 (1956) 1.
[8] D. J. HANAHAN AND M. JAYKO, *J. Am. Chem. Soc.*, 74 (1959) 5070.

THE *STREPTOCOCCUS FAECALIS* OXIDASES FOR REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE

VI. INHIBITION OF THE FLAVOPROTEIN PEROXIDASE BY ITS ELECTRON DONOR

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SUMMARY

When the flavoprotein, DPNH peroxidase, is incubated with DPNH, enzyme activity slowly decays with first-order kinetics ($k = 0.066$ min^{-1}, pH 5.4). The cosubstrate, hydrogen peroxide, can prevent but cannot reverse the inhibition. The kinetics of the inactivation were investigated and shown to be consistent with the following mechanism. (a) DPNH combines with the enzyme to form a binary complex that is kinetically active and has a characteristic absorption spectrum. (b) In the absence of peroxide, the complex is slowly and irreversibly converted to an inactive form. (c) Hydrogen peroxide prevents the inhibition by combining with the binary complex to form a ternary complex that dissociates into products. The ternary complex is not inactivated except at very high DPNH concentrations; this type of inactivation is reversible.

INTRODUCTION

The enzyme DPNH peroxidase is an FAD-containing flavoprotein[1,2] that catalyzes the reaction

$$\text{DPNH} + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{DPN}^+ + 2\text{H}_2\text{O}$$

Previous work from this laboratory showed that the mechanism of the reaction involves the formation of a spectrophotometrically visible complex between DPNH and flavoprotein[2,3]. Evidence that this binary complex is kinetically active has been summarized elsewhere[4,19]. Kinetic data suggest that the binary complex binds peroxide to form a rate-limiting ternary complex, which then decomposes into products[2,4].

In the present report, evidence for a rather unusual type of substrate inhibition is presented. If the oxidant (H_2O_2) in this two-substrate reaction is not present, the reductant (DPNH) causes a slow conversion of the enzyme to an inactive form. The inactivation results either from an irreversible binding of DPNH to the enzyme or

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Abbreviations used: DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; and FAD, flavine adenine dinucleotide.

* Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

from a DPNH-dependent irreversible inactivation of an unspecified nature. Peroxide can arrest or prevent, but it cannot reverse the inactivation. Although many types of substrate inhibition are known[5], the situation just summarized seems not to have been encountered previously for any two-substrate reaction.

METHODS

Assay of inhibited enzyme

The peroxidase assay has been described previously[2]. To follow the kinetics of inactivation, enzyme and DPNH were preincubated in 3-ml cuvettes in the Beckman spectrophotometer, at 24°, in the absence of peroxide. Standard assay conditions were used, except that peroxide was absent and the DPNH level was adjusted to some specified concentration (final volume, 2.9 ml). Concentration profiles (varying DPNH concentration and constant preincubation time) and time profiles (constant DPNH concentration and varying time of preincubation) were determined. At the end of the preincubation period, the active enzyme remaining was assayed by the standard technique. 0.1 ml of a DPNH–H_2O_2 solution was added to bring the DPNH and peroxide concentrations up to the assay level ($8 \cdot 10^{-5}$ *M* DPNH; $1.33 \cdot 10^{-3}$ *M* H_2O_2). On addition of peroxide, the inhibition stopped immediately and the kinetics for DPNH oxidation were zero order.

Materials

Highly purified DPNH peroxidase (minimum molecular weight approximately 120,000, based on the FAD content) was prepared as described previously[1, 2]. DPN and TPN were purchased from Pabst Laboratories. DPNH was prepared from DPN with yeast-alcohol dehydrogenase[6] and TPNH from TPN with the alcohol dehydrogenase of *Leuconostoc mesenteroides*[7]. The reduced nucleotides were isolated and stored as the barium salts[6]. Just prior to use, they were converted to the potassium salts by precipitation of the barium with K_2SO_4.

EXPERIMENTAL

Inhibition of DPNH peroxidase by DPNH

The data of Fig. 1 demonstrate some of the main features of the inactivation phenomenon. Curve A shows that the inactivation obtained by preincubating DPNH and enzyme for 10 min (in the absence of peroxide) is a function of the DPNH concentration in the preincubation mixture. At very low DPNH concentrations, the percentage inactivation is proportional to the DPNH concentration, but at concentrations in excess of $1 \cdot 10^{-6}$ *M*, the inactivation becomes virtually independent of the DPNH level. The percentage inactivation at which saturation occurs depends on the period of preincubation of DPNH and enzyme—the longer the preincubation period, the greater the percentage inactivation obtained at saturation. The kinetics of decay in the region of DPNH saturation are shown in curve B. In a 30-min period, during which 87 % of the initial enzyme activity was lost, the decay of activity followed first-order kinetics. Virtually the entire course of the reaction, therefore, can be described by these simple kinetics and there seem to be no complicating side effects. The results shown in curves A and B suggest that DPNH combines with the flavoprotein to form a complex that, in the absence of peroxide, is slowly converted with first-order kinetics ($k = 0.065\ \text{min}^{-1}$) to an inactive form. The half-saturation value for DPNH determined from curve A ($0.37 \cdot 10^{-6}\ M$) is in good agreement with the dissociation constant determined from a more rigorous mathematical treatment discussed in a later section.

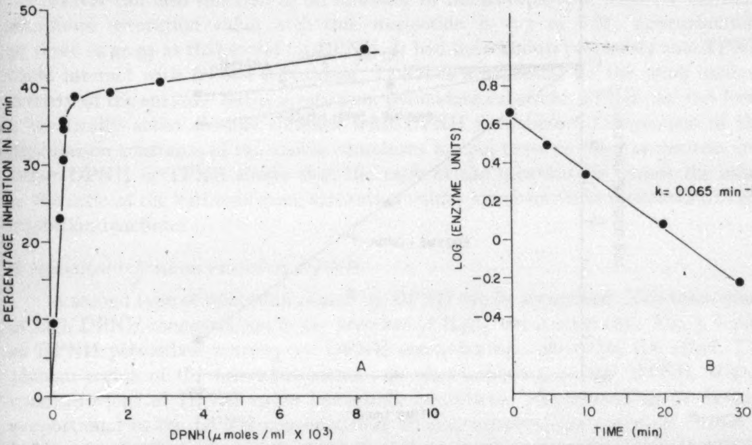

Fig. 1. Inhibition of DPNH peroxidase by DPNH. Standard conditions (METHODS section) were used to measure the activity remaining after preincubation of enzyme and DPNH. Temperature, 24°, pH 5.4. Curve A: DPNH peroxidase, equivalent to $1.5 \cdot 10^{-9}\ M$ bound FAD, incubated 10 min with DPNH in concentrations shown. Volume, 2.9 ml. At the end of the preincubation period, 0.1 ml of a DPNH–H_2O_2 mixture was added to complete the assay system. Curve B: DPNH peroxidase concentration as for curve A, incubated with DPNH, $8.3 \cdot 10^{-5}\ M$. The peroxidase activity remaining at the times indicated was assayed after the addition of H_2O_2, $1.33 \cdot 10^{-3}\ M$ (final concentration).

Protective effect of H_2O_2

As mentioned in the METHODS section, the addition of peroxide to the enzyme–DPNH incubation mixture immediately stops the inactivation reaction. It is this that makes possible the accurate assay of active enzyme in the experiments described here. Hydrogen peroxide can also protect the enzyme against inactivation during prolonged exposure to DPNH. These results are shown in Fig. 2. First, it is evident that incubation of enzyme alone, or enzyme plus peroxide, causes only a small loss in activity. When the flavoprotein is exposed to DPNH, activity decays in the usual manner; however, in the presence of enough peroxide to ensure that the flavoprotein is all in the form of its ternary complex (composed of enzyme, DPNH, and peroxide) most of the decay in activity is prevented. The loss in activity is a little greater than that which takes place in the presence of peroxide alone. In this experiment, the addition of peroxide completes the assay system, and DPNH oxidation takes place at a constant rate. During the entire time course of the oxidation, however, the DPNH level was deliberately maintained above saturation (for both the inactivation and peroxidase reactions) so that the protective effect of peroxide cannot be ascribed to a decrease in the DPNH concentration. The data are in accord with the hypothesis that peroxide exerts its protective effect by combining with the binary complex of flavoprotein and DPNH to form a ternary complex that does not undergo inactivation.

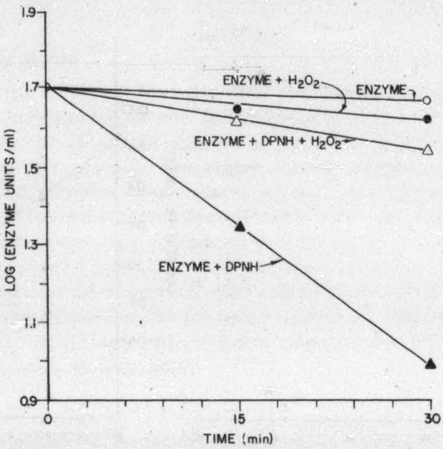

Fig. 2. Inactivation of DPNH peroxidase: protection by H_2O_2. Enzyme, equivalent to $0.89 \cdot 10^{-9}$ *M* bound FAD, was incubated with the components indicated, and at 15 and 30 min, enzyme assays were performed (standard conditions). ○, DPNH peroxidase; ●, DPNH peroxidase + H_2O_2, $1.8 \cdot 10^{-3}$ *M*; △, DPNH peroxidase + DPNH + H_2O_2 (DPNH concentration was maintained above $5 \cdot 10^{-5}$ *M* during the course of the experiment; H_2O_2, $1.8 \cdot 10^{-3}$ *M*); ▲, DPNH peroxidase + DPNH, $2.2 \cdot 10^{-5}$ *M*.

Attempts to reverse the inactivation

All attempts to reverse the inhibition brought about by DPNH have been unsuccessful. The inhibited enzyme is neither reactivated by dialysis nor by incubation with the known flavins, with hot-water extracts of *Streptococcus faecalis* cells, or with the various metal ions. Fluorometric[8] and enzymic analysis[9] show that there is no detectable difference in the FAD content of active and DPNH-inhibited enzyme.

Substrate specificity for inactivation

Neither DPN nor the primary acid modification product of DPNH is inhibitory[10]. The loss of inhibitory activity on conversion of DPNH to the modification product (presumably the trihydromonohydroxy nicotinamide analogue of DPN[11]) is evidence that the inhibitor is DPNH itself and not an incidental contaminant of the DPNH preparation. To obtain further information on this point, the following experiment was performed. DPNH was prepared from 95 % pure DPN with alcohol dehydrogenase. Two control reaction mixtures were run through the entire alcohol dehydrogenase procedure, one without alcohol dehydrogenase and one without DPN. After the DPN in the complete system had been converted to DPNH, the three reaction mixtures were heated at 100° for 3 min, cooled to 0°, and centrifuged. When aliquots of the DPNH-containing reaction mixture were tested for their inhibitory effect they gave a saturation curve identical, within experimental error, to the one shown in Fig. 1. Aliquots from the two control reaction mixtures were not inhibitory. The results, therefore, indicate that the true inhibitor is the reduced nucleotide.

TPNH can also function as an inhibitor of the flavoprotein, however the half-maximum saturation value with this nucleotide is $3.3 \cdot 10^{-5}$ *M*, approximately 90 times as great as that found for DPNH. It had been known previously that TPNH could interact with DPNH peroxidase; TPNH is a substrate for the weak oxidase activity of the enzyme[2] but is a very poor peroxidase substrate. TPNH can also form a kinetically active, visible complex with DPNH peroxidase[4]. Comparison of the dissociation constants of the visible complexes formed between the flavoprotein and either DPNH or TPNH shows that the ratio of the constants is almost the same as the ratio of the half-maximum saturation values found for these nucleotides in the inhibition reaction.

A reversible inhibition caused by DPNH

A second type of inhibition caused by DPNH can be recognized. This takes place at high DPNH concentration in the presence of H_2O_2 and is reversible. Fig. 3, a plot of DPNH peroxidase activity *vs.* DPNH concentration, illustrates the effect. The plateau region of the saturation curve extends to about $5 \cdot 10^{-4}$ *M* DPNH. Higher concentrations of DPNH cause immediate inhibitions, which seem to be directly proportional to the DPNH concentration. In this region of the curve, as DPNH is used up in the peroxidase reaction, the reaction rate increases, and when the DPNH concentration falls below $5 \cdot 10^{-4}$ *M*, the velocity is almost at the experimentally obtainable maximum. The half-maximum saturation value for DPNH as an inhibitor in this reaction is about 55 times as great as its half-maximum saturation value as a substrate. The reversible inhibition demonstrated here may reflect the binding of an additional molecule of DPNH to the ternary complex.

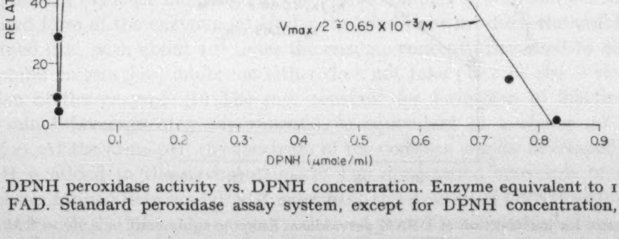

Fig. 3. DPNH peroxidase activity vs. DPNH concentration. Enzyme equivalent to $1.32 \cdot 10^{-9}$ *M* bound FAD. Standard peroxidase assay system, except for DPNH concentration, as shown.

Test of a proposed mechanism for the inhibition reaction

The data that have been presented thus far suggest the following mechanism for the irreversible inhibition and its prevention by peroxide:

$$\begin{tikzcd}
E + DPNH \arrow[r, shift left, "k_1"] & E\cdot DPNH \arrow[l, shift left, "k_2"] \arrow[r, "k_3"] \arrow[d, shift left, "H_2O_2"] & EI \\
& E\cdot DPNH\cdot H_2O_2 + H^+ \arrow[u, shift left, "-H_2O_2"] \arrow[r, "k_6"] & E + DPN^+ + 2H_2O
\end{tikzcd}$$

Enzyme and DPNH combine to form the binary complex E·DPNH. This is the complex that has the characteristic absorption spectrum previously dexscribed[2,3] and normally would combine with peroxide to form the ternary complex $E\cdot DPNH\cdot H_2O_2$. Decomposition of the latter complex yields DPN and $2H_2O$. If, however, peroxide is not present, the binary complex is slowly converted to the inactive form, EI, in an irreversible step. The following equation is a linear form of the integrated rate equation for the proposed mechanism (inactivation in the absence of peroxide):

$$\frac{1}{\ln [E_0/(E_0 - EI)]} = \frac{1}{k_3 t} + \frac{1}{S}\left(\frac{k_2}{k_1} \times \frac{1}{k_3 t}\right)$$

Where E_0 is total enzyme (initial enzyme activity); EI, inactive enzyme (initial enzyme activity minus activity remaining at time t); and S, DPNH concentration*.

A plot of the reciprocal of $\ln[E_0/(E_0 - EI)]$ *vs.* $1/S$ is a straight line, with the y intercept equal to $1/k_3 t$ and the slope equal to $k_2/k_1 \times 1/k_3 t$. Thus, the first-order rate constant, k_3, and the dissociation constant, k_2/k_1, can be obtained from this treatment. The experimental data necessary for testing the mechanism can be obtained by

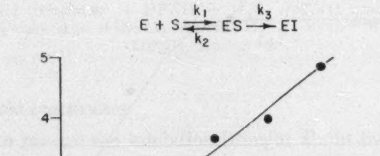

Fig. 4. Kinetics for inactivation of DPNH peroxidase. Enzyme equivalent to $1.66\cdot 10^{-9}$ *M* bound FAD, incubated 10 min with DPNH in concentrations shown (sodium acetate buffer pH 5.4, 0.033 *M*) and then assayed in standard assay system.

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* The author is indebted to Dr. W. A. Arnold, Biology Division, Oak Ridge National Laboratory, for deriving this rate equation. In the derivation, it was assumed that the value of k_3 (0.066 min^{-1}) is negligible when compared with the magnitude of the other rate constants.

determining the amount of active enzyme left after a given period of exposure of enzyme to a series of DPNH concentrations. Fig. 4 demonstrates that the experimental data are consistent with the proposed mechanism, at least over a 40-fold range of DPNH concentrations. The rate constant for inactivation ($k_3 = 0.069\ \text{min}^{-1}$) is in good agreement with the constant evaluated by following enzyme decay at saturating DPNH concentrations (Fig. 1). A summary of two experiments is shown in Table I. One set of data was obtained under conditions in which the enzyme shows its maximum activity (pH 5.4, acetate buffer) and the other under conditions in which peroxidase activity is low (pH 7.1, phosphate buffer). It is interesting that there is a reciprocal relation between the constants k_3 and k_6. Change of conditions from pH 7.1 (phosphate) to pH 5.4 (acetate) causes an eightfold increase in k_6 and a fivefold decrease in k_3. That is, the very conditions that cause a more rapid dissociation of the ternary complex into products also aid in maintaining the binary complex in an active form.

TABLE I

KINETIC CONSTANTS FOR DPNH PEROXIDASE

Conditions as for Fig. 4, except that for the experiment at pH 7.1, potassium phosphate (0.066 *M*) was the buffer in both the preincubation and assay phases of the experiment.

pH	k_2/k_1 *(Mole/liter)*	k_3 *(min⁻¹)*	k_6 *(min⁻¹)*
5.4	$0.66 \cdot 10^{-6}$	0.069	$6 \cdot 10^3$
7.1	$1.9 \cdot 10^{-6}$	0.34	$0.78 \cdot 10^3$

The ratio k_2/k_1 should be the true dissociation constant of the E·DPNH complex. As might be expected, this value is lower than the Michaelis constants obtained when either peroxide of ferricyanide serve as oxidants for DPNH oxidation[2]. Estimates for some of the individual rate constants in the mechanism just postulated have been given elsewhere[4].

Relation between the inhibited form of the enzyme and the visible complex

When substrate amounts of DPNH peroxidase are used, it can be demonstrated that the enzyme forms a spectrophotometrically visible complex with DPNH[2,3]. Three lines of evidence indicate that the visible complex is not identical with EI, the inhibited form of the enzyme. (a) Under the conditions in which the visible complex is formed (*i.e.*, with about 10^4 times the enzyme concentration used to demonstrate the inhibition reaction) inhibition either does not take place or else is reversible on dilution of the enzyme. (b) The rate constant for formation of inactive enzyme, $0.066\ \text{min}^{-1}$ (average of 5 experiments), is equivalent to a change of 6.4 %/min (pH 5.4). At the same pH, the spectrum of the complex is fully developed as soon as DPNH is added to the flavoprotein. (c) The dissociation constants of the visible complexes formed between DPNH peroxidase and a series of DPNH analogues agree very well with the Michaelis constants derived from kinetic studies[4]. This correlation is strong evidence that the visible complex is kinetically active.

Evidence on the first point was obtained by incubation of DPNH peroxidase ($7 \cdot 10^{-5}\ M$) with an excess of DPNH under conditions that would have caused 70 % inhibition of dilute enzyme solutions. At the end of the incubation period, the enzyme was diluted in peroxide-containing buffer (H_2O_2 was present to prevent the DPNH-dependent inactivation that takes place at low enzyme concentrations) and assayed for peroxidase activity. There was no detectable loss in activity. It seems, therefore, that, at high enzyme concentrations, the peroxidase is able to protect itself against the inactivation. It is possible that dilution of the enzyme causes a slight conformation change that makes the enzyme susceptible to substrate inhibition, whereas at high protein concentration, the enzyme conformation might prevent such inhibition. The data of Table I suggest that the binary complex may be stabilized in an appropriate environment.

DISCUSSION

The inhibition that has been described does not seem to be identical with any reported previously, although there are superficial similarities to other systems. For instance, the active catalase·H_2O_2 complex (complex I) can be slowly, but reversibly, transformed into the inactive complex II under conditions in which the peroxide concentration is maintained at a low, steady-state level for extended periods of time[12]. Certain copper-containing oxidases are progressively inactivated when they function as catalysts[13]. The kinetics of this "reaction inactivation" have been intensively investigated[13,14]. Among the flavoproteins, the DPNH oxidase of *Clostridium perfringens* undergoes a type of inhibition more nearly like the one described in the present paper[15]. The oxidase is slowly inhibited by DPNH in the absence of the oxidant (O_2). Inhibition, however, also takes place when the enzyme is "turning over". Under conditions in which the oxidase catalyzes DPNH oxidation, oxidase activity decays with first-order kinetics. Unlike DPNH peroxidase, the oxidase of *C. perfringens* is very sensitive to peroxide; the rate constant for inactivation of the oxidase increases as the peroxide level is raised.

Thus there are several examples of substrate-induced slow decay of enzyme activity not attributable to product formation. The interesting feature of the DPNH peroxidase system is that the decay is almost completely prevented by the cosubstrate, peroxide. This circumstance makes possible a complete separation of the kinetics of inactivation from those of substrate turnover. As previously discussed, the results of kinetic studies are consistent with a simple mechanism—namely, that DPNH combines with the enzyme to form a binary complex that is initially kinetically active but in the absence of peroxide is slowly converted to an inactive form. Peroxide prevents the inhibition by maintaining the enzyme in the form of its ternary complex. The cause of the inhibition is unknown. Since the inactivation is not reversed by dialysis, it may be that DPNH becomes very tightly bound to the enzyme and can no longer function as a substrate, however, there are obviously alternative interpretations. For instance, the binding of small molecules to protein or of substrate to enzyme can apparently cause conformation changes in the protein[16–18]. The manner in which a change in conformation may enhance enzyme activity has been discussed[16]. For certain two-substrate reactions, it is conceivable that a conformation change induced in the enzyme by one substrate could make the enzyme liable to denaturation unless the cosubstrate were present. It would be interesting to know whether any other two-substrate reactions show the behavior described for the DPNH-peroxidase system.

REFERENCES

[1] M. I. DOLIN, *Arch. Biochem. Biophys.*, 55 (1955) 415.
[2] M. I. DOLIN, *J. Biol. Chem.*, 225 (1957) 557.
[3] M. I. DOLIN, *Arch. Biochem. Biophys.*, 60 (1956) 499.
[4] M. I. DOLIN, in I. C. GUNSALUS AND R. Y. STANIER, *The Bacteria*, Vol. II, Academic Press Inc., New York, 1960, in the press.
[5] J. M. REINER, *Behavior of Enzyme Systems*, Burgess Publishing Co., Minneapolis, 1959.
[6] G. W. RAFTER AND S. P. COLOWICK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press Inc, New York, 1957, p. 887.
[7] R. D. DEMOSS, *J. Bacteriol.*, 68 (1954) 252.
[8] O. A. BESSEY, O. H. LOWRY AND R. H. LOVE, *J. Biol. Chem.*, 180 (1949) 755.
[9] C. DELUCA, M. M. WEBER AND N. O. KAPLAN, *J. Biol. Chem.*, 223 (1956) 559.
[10] G. W. RAFTER, S. CHAYKIN AND E. G. KREBS, *J. Biol. Chem.*, 208 (1954) 799.
[11] A. G. ANDERSON AND G. BERKELHAMMER, *J. Am. Chem. Soc.*, 80 (1958) 992.
[12] B. CHANCE, *Biochem. J.*, 46 (1950) 387.
[13] C. R. DAWSON, in W. D. MCELROY AND B. GLASS, *A Symposium on Copper Metabolism*, The Johns Hopkins Press, Baltimore, 1950, p. 18.
[14] L. L. INGRAHAM, J. CORSE AND B. MAKOWER, *J. Am. Chem. Soc.*, 74 (1952) 2623.
[15] M. I. DOLIN, *J. Bacteriol.*, 77 (1959) 383.
[16] R. LUMRY AND H. EYRING, *J. Phys. Chem.*, 58 (1954) 110.
[17] J. T. EDSALL, *Discussions Faraday Soc.*, 13 (1953) 9.
[18] S. SHIFRIN, N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 234 (1959) 1555.
[19] M. J. DOLIN, *J. Biol. Chem.*, 235 (1960) 544.

THE EXTENT OF ENERGY MIGRATION AND CHLOROPHYLL *A* ORIENTATION IN CHLORELLA

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SUMMARY

1. A method is described by which $\bar{n}$, the average number of energy transfers between different chlorophyll *a* molecules during the mean lifetime of the excited state, can be measured *in vivo*. The effect produced by added quencher molecules on the fluorescence excitation spectrum and yield enables $\bar{n}$ to be calculated without assumptions regarding the mutual orientation of the pigment molecules.

2. The calculated mean value of $\bar{n}$ was 275. Although subject to considerable errors, this value is comparable with $\bar{n}_r$ measured in Chlorella and in concentrated chlorophyll solutions by fluorescence depolarization. Because $\bar{n}_r$ is calculated on a basis of random molecular orientation, the chlorophyll *a* molecules *in vivo* cannot possess a high degree of preferred orientation.

3. The time interval occupied by each transfer process is calculated, together with the trapping efficiencies to be expected *in vivo*.

INTRODUCTION

In photosynthesis, resonance energy transfer appears to play an essential part in conveying energy derived from light absorption by pigment molecules to photochemically active centres present in relatively small concentration. This energy transfer is marked by the sensitization of both photosynthesis and chlorophyll *a* fluorescence by accessory pigments[1, 2], and the low values of fluorescence quantum yield[3] and polarization[4] shown by chloroplasts.

The extent of energy migration among chlorophyll molecules in solution can be calculated from the observed fluorescence polarization of concentrated solutions in viscous media which prevent molecular rotations during the lifetime of the singlet excited state. The relationship

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3}{2}\,\overline{\mathrm{Sin}^2\theta}\cdot\bar{n}\right) \tag{1}$$

derived by WEBER[5], relates the observed polarization p to that in the absence of energy transfer p_0, the angle between the emission oscillators θ, and the average number of energy transfers $\bar{n}$ between different molecules before emission. For randomly oriented molecules, the case in solution, $\mathrm{Sin}^2\theta = 0.404$, so that the equation (1) becomes

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3}{5}\cdot\bar{n}_r\right) \tag{2}$$

In systems which may not be randomly oriented, such as chlorophyll *in vivo*, $\bar{n}_r$ represents the minimum number of random transfers required to produce the observed depolarization. Since $\bar{n}$ represents an average value, in systems quenched by a process of energy migration to trapping centres the observed fluorescence will result from the shorter transfer sequences, and equation (2) becomes

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{\phi}{\phi_0}\,\frac{3}{5}\,\bar{n}_r\right) \tag{3}$$

where ϕ_0 is the fluorescence yield of the unquenched system, and ϕ the reduced yield produced by transfer quenching. Application of equation (3) in the case of chloroplast fluorescence, providing the appropriate value for ϕ/ϕ_0 is known, permits the calculation of $\bar{n}_r$, a minimal value which may be equivalent to more extensive energy migration in a system having some preferred orientation.

On the other hand, the fluorescence quenching produced by energy transfer to trapping centres is given by the relationship

$$\frac{\phi_0}{\phi}=\frac{1+K\bar{n}T}{1-T} \tag{4}$$

where T is that fraction of all the molecules which act as trapping centres having a relative probability of transfer K from the fluorescent species, compared with unit probability of transfer between the fluorescent species. K is related to the integrated overlap between the fluorescence spectrum of the donor molecule and the molecular absorption spectrum of the trapping molecule. $\bar{n}$ is the average number of transfers between different molecules without assumptions regarding mutual orientation.

Providing the quantities T and K are known in a system quenched by transfer to trapping centres, the value of $\bar{n}$ can be calculated, and if $\bar{n}_r$ is known the average angle between molecules is given by

$$\overline{\mathrm{Sin}^2\theta} = \frac{2\,\bar{n}_r}{5\,\bar{n}} \tag{5}$$

When additional trapping centres T_2 are added to a partly quenched system, the equation (4) can be written

$$\frac{\phi_0}{\phi_2} - \frac{\phi_0}{\phi_1} = \bar{n}K_2T_2 \tag{6}$$

where ϕ_1 and ϕ_2 are the fluorescence yields before and after the addition of trapping centres T_2 with transfer probability K_2, and where the total fraction of trapping molecules is small compared with unity.

In practice the number of trapping centres in chloroplasts can be increased by suspending the material in aqueous solutions of aromatic nitrocompounds[6] which quench chlorophyll fluorescence. In most cases no changes in chlorophyll absorption take place, so that $K_2 = 1$. The value of T_2, the fraction of quenched chlorophyll molecules produced by the nitrocompound cannot be calculated from the concentration of the aqueous solution employed to produce quenching. Fortunately in Chlorella the occurrence of chlorophylls *a* and *b*, which are both quenched by nitrocompounds, enables T_2 to be measured by the following considerations. The appearance of the fluorescence spectrum characteristic of chlorophyll *a* when Chlorella is excited by radiation absorbed predominantly by chlorophyll *b* indicates efficient energy transfer between these pigments, which has also been demonstrated *in vitro*. Providing the probability of transfer between chlorophyll *b* molecules is negligible compared with that for transfer to chlorophyll *a*, then we can represent the chlorophyll transfers *in vivo* by the scheme

$$\begin{tikzcd} b \arrow[r] & {[a]} \arrow[r, "\bar{n}"] & \text{Fluorescence or trapping} \\ \lambda_b \arrow[u] & \lambda_a \arrow[u] & \end{tikzcd}$$

where λ_b and λ_a are radiation wavelengths exciting chlorophyll *b* and *a* respectively. The relative fluorescence yields ϕ_a and ϕ_b of the two chlorophylls can be written

$$\phi_a = \frac{1 - T_2}{1 + \bar{n}T_2} \qquad \phi_b = \frac{(1 - T_2)^2}{1 + \bar{n}T_2}$$

assuming that the nitrocompound is distributed uniformly and quenches a similar fraction of both chlorophylls. The ratio of the relative yields is $\phi_b/\phi_a = (1 - T_2)$, so that T_2 can be measured in terms of the change produced by quenching in the relative yields of chlorophylls *a* and *b*, together with change in ϕ_a. The value of $\bar{n}$ is given by the relationship

$$\bar{n} = \frac{(\phi_0/\phi_{a'})\,(\phi_b/\phi_a) - 1}{1 - \phi_b/\phi_a} \tag{7}$$

where ϕ_0 is the chlorophyll *a* yield in the absence of any quenching, and $\phi_{a'}$ the yield after the addition of the quenched fraction T_2.

EXPERIMENTAL

The most suitable quencher for use with Chlorella was found to be *m*-dinitrobenzene, which penetrated the chloroplasts rapidly and reversibly without producing detectable absorption or chemical changes. The polarization of the fluorescence of the chlorophylls in organic solvents was unaltered by quenching, suggesting that quenching results from the formation of a weak complex in the ground state, undetectable in absorption measurements. This conclusion was supported by the decreased quenching efficiency observed at higher temperatures.

Fluorescence measurements were made using a xenon arc lamp together with a grating monochromator to select exciting radiation of the desired wavelengths. The red fluorescence from the specimen cell was observed normally to the excitation direction through a glass filter (Corning 5850) which transmitted wavelengths longer than 680 mμ, and was detected with a red-sensitive photomultiplier (RCA 6217). In a series of readings with graded concentrations of quencher, equal amounts of Chlorella were used, so that the optical geometry was similar for all readings. At each excitation wavelength, the cell blank was eliminated by measuring the fluorescence intensity at progressive dilutions of the original cell suspension, and extrapolating to zero concentration. The wavelengths λ_b and λ_a were chosen as 480 mμ and 670 mμ respectively, and at each quencher concentration the relative yield ϕ_a was recorded, together with the ratio ϕ_b/ϕ_a.

Fluorescence polarization measurements were made with the polarization photometer described by WEBER[7]. Solutions of chromatographically pure pigments in cyclohexanol, having a maximum concentration of $8 \cdot 10^{-3}$ *M*, were examined in very thin layers[8] to avoid reabsorption effects.

RESULTS

In Fig. 1 typical plots of $\phi_a/\phi_{a'}$ and ϕ_b/ϕ_u against quencher concentration in the aqueous phase are shown for a specimen of Chlorella having an absolute quantum yield of chlorophyll ϕ_a of 2.7 % before quenching. Similar plots of the results of many experiments showed that the ratio ϕ_b/ϕ_a decreased by an average of 11 % for a four-fold decrease in ϕ_a. The degree of quenching produced by constant concentrations of *m*-dinitrobenzene was independent of the amount of Chlorella employed, showing that the amount taken up from solution is very small.

In order to calculate the relative transfer probabilities of the chlorophylls, the fluorescence excitation and fluorescence spectra were measured and are shown in Fig. 2. The excitation spectrum of chlorophyll *a* was derived from that of Chlorella by considerations of the band width and band maximum of the red fluorescence, and the excitation spectrum of the chlorophyll *b* component obtained by difference. The position assigned to the fluorescence spectrum of the latter pigment is tentative, but has been related to its excitation spectrum in the same manner as for chlorophyll *a*.

The fluorescence polarization of Chlorella excited with radiation of 660 mμ wavelength was 0.06 ± 0.01. In Fig. 3 the effect of concentration on the fluorescence polarization of chlorophylls *a* and *b* in cyclohexanol is shown. Cd 644 mμ radiation was used to excite fluorescence.

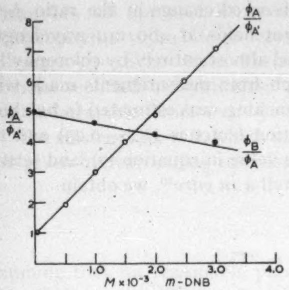

Fig. 1.

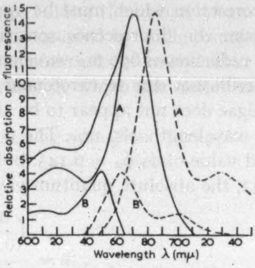

Fig. 2.

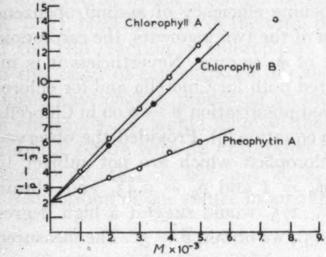

Fig. 3.

Fig. 1. The change in chlorophyll *a* yield expressed as the ratio ϕ_a/ϕ_a' and the chlorophyll *b* yield expressed as the ratio ϕ_b/ϕ_a produced by *m*-dinitrobenzene quenching of Chlorella.

Fig. 2. The fluorescence and fluorescence excitation spectra of Chlorella, and the component chlorophylls. ----, fluorescence spectra; ——, fluorescence excitation spectra.

Fig. 3. The effect of concentration on the fluorescence polarization of concentrated solutions of chlorophylls *a* and *b* and pheophytin *a* in cyclohexanol.

DISCUSSION

According to the theory developed by FÖRSTER[9], the relative probability of transfer between chlorophyll *b* molecules compared with the transfer to chlorophyll *a* is given by the ratio

$$\frac{Pb\longrightarrow b}{b\longrightarrow a}=\frac{R^6_{ba}}{R^6_{bb}}\cdot\frac{\int_0^\infty I_\lambda\cdot b_\lambda}{\int_0^\infty I_\lambda\cdot a_\lambda}$$

where $R_{b\text{-}a}$ and $R_{b\text{-}b}$ are the average intermolecular separations of the respective chlorophyll molecules, and the integrals represent the integrated overlaps between the chlorophyll *b* fluorescence spectrum and the molecular absorption spectra of chlorophylls *a* and *b* respectively. This latter quantity was estimated from Fig. 2 as approximately 6. Substituting this value, and the relative concentration of the two pigments in the Chlorella, which had a molecular ratio $a:b = 3:1$, we have

$$\frac{Pb\longrightarrow b}{b\longrightarrow a}=\frac{1}{(3+1)^2}\times\frac{1}{6}=0.01$$

This low relative probability of transfer means that excited chlorophyll *b* molecules transfer directly to neighbouring chlorophyll *a* molecules, and the assumptions made in the derivation of equation (7) appear justified.

A correction which must be applied to the observed change in the ratio ϕ_b/ϕ_a arises from the fluorescence sensitization by carotenoids at 480 mμ wavelength, whereas radiation of 670 mμ wavelength is absorbed almost entirely by chlorophyll *a*. The contribution due to carotenoid transfer, which from measurements made with brown algae does not appear to be affected by quenching, was estimated to be about 23 % at wavelength 480 mμ. The required correction factor is $1/(1-0.23)$ and the corrected value of $\phi_b/\phi_a = 0.143$. Substituting this value in equation (7), and setting $\phi_0 = 0.32$, the absolute quantum yield of chlorophyll *a in vitro*[10], we obtain

$$\bar{n} = \frac{\left(\frac{0.32}{0.027} \times 4\right) - 1}{0.143} = 275$$

The errors involved in the measurement of $\bar{n}$ depend on many factors, some of which cannot be evaluated, such as the relative quenching efficiency of *m*-dinitrobenzene for the two chlorophylls *in vivo*, the distribution of the two pigments, the carotenoid contribution, and the uncertainty of the value of ϕ_0 *in vivo*. Nevertheless it is instructive to compare this value with $\bar{n}_r$, calculated both for Chlorella and for chlorophyll solutions. The interpretation of the observed polarization $p = 0.06$ in Chlorella depends on the value assigned to the ratio ϕ/ϕ_0 in equation (3). Provided the observed fluorescence originates from regions in the chloroplast which are not subject to competitive transfer quenching, we can set $\phi/\phi_0 = 1$ and $p_0 = 0.43$, and obtain $\bar{n}_r = 12$. This small value, compared with $\bar{n} = 275$, would suggest a high degree of molecular orientation, and applying equation (5), we obtain $\bar{\theta} = 5°$. The measured dichroism[11] of chloroplasts is certainly lower than would be expected from this figure, but could be lowered by structural features above the pigment molecular level. On the other hand, most probably the observed fluorescence of Chlorella results from a competitive quenching process which reduces the fluorescence yield from 0.32 to 0.027, so that $\phi/\phi_0 = 0.085$ and we obtain $\bar{n}_r = 141$. This value is more comparable with $\bar{n}$, and taken together indicate only a low degree of orientation. Moreover, this value of $\bar{n}_r$ is similar to those measured in concentrated solutions of chlorophyll *a*, on extrapolation to concentrations known to exist in the chloroplast[12]. Using the data shown in Fig. 3, $\bar{n}_r$ is 8.9 in a $5 \cdot 10^{-3}$ *M* solution of chlorophyll *a*. At this concentration, no concentration quenching took place, and from this value of $\bar{n}_r$ the intermolecular distance at which emission and transfer are equally probable can be calculated at 35 Å. Since $\bar{n}_r$ is linear with concentration in the absence of quenching, by extrapolation $\bar{n}_r$ would be 177 in 10^{-1} *M* solution. The similarity of this value to those for $\bar{n}$ and $\bar{n}_r$ in Chlorella means that concentration quenching must be negligible *in vivo*, despite the existence of chlorophyll concentrations sufficient to produce very strong quenching when observed in solutions of chlorophyll in organic solvents. Formation of non-fluorescent dimers which act as trapping centres undoubtedly produces concentration quenching, and presumably an efficient mechanism exists in the chloroplast which prevents this molecular association.

Both $\bar{n}$ and $\bar{n}_r$ measure the number of different molecules visited during the transfer sequence. Repeated exchanges between the same molecular pair produce no change in observed polarization or quenching provided no changes occur in molecular orientation or the distribution of quenched complexes during the fluorescence lifetime. The transfer process must proceed by a random walk, so that the actual number of transfers will approach $\bar{n}^2$, or from $2.0\text{–}7.6 \cdot 10^4$. Taking the actual mean lifetime of chlorophyll *a* to be $6.6 \cdot 10^{-9}$ second[13], the time occupied by each transfer lies in the range $3.3\text{–}0.8 \cdot 10^{-13}$ sec. Transfer during time intervals of comparable brevity has been reported between close pairs of molecules in which the donor molecules have undetectable fluorescence yield[14,15].

The efficiency E, with which quanta are trapped by a fraction of trapping centres T, is given by rearranging equation (4),

$$E = \frac{K\bar{n}T}{1 + K\bar{n}T}$$

Assuming that for reasonable photosynthetic efficiency $E \geqslant 0.75$, at this arbitrary value $K\bar{n}T = 3$ or $KT = 3/\bar{n}$. For $\bar{n} = 275$ the required trapping efficiency would be achieved by trapping centres having the same absorption spectrum as chlorophyll *a* ($K = 1$), present as 1.1 of the total pigment. The change in the photochemical yield of the Hill reaction in chloroplasts with progressive fragmentation[16,17] also points to this order of concentration for the trapping centre. An absorption maximum at longer wavelengths, producing an increased overlap with the fluorescence spectrum of chlorophyll *a*, would increase the trapping efficiency in proportion to K. For a trapping centre with an absorption band coincident with the chlorophyll *a* fluorescence band ($\lambda_{\text{max.}} = 687\ \text{m}\mu$), $K \simeq 3$ and high efficiencies could be achieved with a distribution of one centre in an average of 300 chlorophyll molecules.

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REFERENCES

[1] L. N. M. Duysens, *Thesis*, University of Utrecht, 1952.
[2] H. J. Dutton, W. M. Manning and B. M. Duggar, *J. Phys. Chem.*, 47 (1943) 308.
[3] P. Latimer, T. T. Bannister and E. Rabinowitch, *Science*, 124 (1956) 585.
[4] W. Arnold and E. S. Meek, *Arch. Biochem. Biophys.*, 60 (1956) 82.
[5] G. Weber, *Trans. Faraday Soc.*, 50 (1954) 552.
[6] R. Livingston and C-L. Ke, *J. Am. Chem. Soc.*, 72 (1950) 909.
[7] G. Weber, *J. Opt. Soc. Am.*, 46 (1956) 962.
[8] G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, 54 (1958) 640.
[9] Th. Förster, *Fluorescenz organischer Verbindungen*, Vandenhoeck and Ruprecht, Göttingen, 1951.
[10] G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, 53 (1957) 646.
[11] J. C. Goedheer, *Biochim. Biophys. Acta*, 16 (1955) 471.
[12] E. Rabinowitch, *Photosynthesis*, Vol. 1, Interscience Publ. Inc., New York, 1945, p. 412.
[13] S. S. Brody and E. Rabinowitch, *Science*, 125 (1957) 555.
[14] G. Weber, *Nature*, 180 (1957) 1049.
[15] F. W. J. Teale, *Nature*, 181 (1958) 415.
[16] J. B. Thomas, O. H. Blaauw and L. N. M. Duysens, *Biochim. Biophys. Acta*, 10 (1953) 230.
[17] J. B. Thomas, O. H. Blaauw and L. N. M. Duysens, *Biochim. Biophys. Acta*, 9 (1952) 575.

STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

X. DISAPPEARANCE OF HYDRAZINE FROM CULTURES OF *A. VINELANDII*

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SUMMARY

The effect of hydrazine on the growth of *Azotobacter vinelandii* has been studied. It was found that small inocula fail to multiply in media containing hydrazine and that addition of hydrazine to proliferating cultures arrests growth. Both inhibitory effects are reversible.

The rate of hydrazine disappearance from cultures of *A. vinelandii* depends directly on the density of the culture and is independent of initial concentration. The disappearance is due to the formation of a non-toxic, acid labile, hydrazine compound which accumulates in the medium surrounding the cells. Two coloured reaction products with picryl chloride, believed to be the picryl derivatives of this hydrazine compound, and a small amount of pyruvic acid were also isolated from such culture supernatants.

INTRODUCTION

One of the most important aspects of the problem of biological nitrogen fixation is the identification of the intermediate compounds and reactions through which molecular nitrogen is transformed into ammonia and thence into organic nitrogen compounds. If a reaction scheme is envisaged, in which all the individual steps are reductive, then hydrazine must be an intermediate, thus,

$$N_2 \xrightarrow{2H} \underset{\text{"diimide"}}{[NH = NH]} \xrightarrow{2H} \underset{\text{hydrazine}}{NH_2\text{–}NH_2} \xrightarrow{2H} 2NH_3$$

This theory led to investigations of the effects of hydrazine on *Azotobacter*[1-3] and other fixers[4]. BURK AND HORNER[1] have shown that it inhibits growth in *A. vinelandii* and that it cannot be utilised as a source of nitrogen by the organism. However, AZIM[2,5] found that cultures of *Azotobacter* caused hydrazine to disappear from the surrounding medium at rates greater than could be accounted for by aerial oxidation. Recently BACH[3] using heavy suspensions of resting cells observed a similar interaction.

This investigation is a re-examination of the phenomenon of "disappearance" and the changes brought about in *A. vinelandii* cultures by the addition of small amounts of hydrazine.

EXPERIMENTAL

Analytical

Hydrazine: Two methods were used to estimate hydrazine in the supernatant culture liquids. The azine method is based on the formation of a yellow reaction product with *p*-dimethylaminobenzaldehyde in aqueous alcoholic solutions under acid conditions[6]. The procedure used was that of WALT AND CHRISP[7]. Optical densities were measured on a Hilger "Spekker" photoelectric absorptiometer using Bright Spectrum Filter 601 and 1-cm cells. Beer's law is obeyed over the range 0 to 2.0 p.p.m., slight deviations becoming apparent at higher concentrations. The picryl chloride method is based on the reaction of hydrazine with picryl chloride to give hexanitrohydrazobenzene[8] which in weakly alkaline solutions assumes an intense violet colour. CHERKESSOV AND KUL'BERG[9] and RILEY[10] used this reaction to develop colorimetric methods but neither of these two methods was suitable in our case; the first makes use of a heterogeneous system in which the reagent is present in a fine suspension, whilst the second utilises non-aqueous solvents which cause the salts present in the bacteriological medium to precipitate out. The following procedure was adopted.

Reagent: 0.2 % w/v ethanolic picryl chloride. Freshly prepared solutions from pure material were colourless.

Buffer: Phosphate buffer of pH 7.28. Phosphate = 1 *M* (KH_2PO_4 = 3.81 %; K_2HPO_4 = 12.54 %).

Procedure: 4 ml of the test solution and 1 ml of buffer were placed in a 10-ml graduated flask. 5 ml of the reagent were added slowly and with shaking. The contraction in volume was made up with distilled water and the colour intensity measured after 5 min. It is important that the reagent is added to the buffer and not the other way round, as sudden mixing causes the picryl chloride to come out of solution as an oil. The pH of the solution, measured with a glass electrode, is 8.35 to 8.37, *i.e.* about 1 pH unit higher than that of the aqueous buffer. Under these conditions the colour develops within 5 and is stable for a further 25 min. Optical densities were measured on a Unicam S.P. 300 G.P. absorptiometer using a green filter.

Total hydrazine: Total hydrazine (*i.e.* free plus acid labile) was determined by the azine method after hydrolysing the sample in 3 *N* hydrochloric acid at 100° for 15 min. The hydrolysate was then diluted with distilled water until it was 1 *M* with respect to acid, the concentration required for the azine method.

Pyruvic acid: The pyruvic acid which accumulates in the supernatant culture liquids during hydrazine disappearance was estimated as 2,4-dinitrophenylhydrazone, according to the general procedures for the determination of α-keto acids[11, 12]. In these methods ethyl acetate is commonly used to extract the hydrazones from the acid reaction mixture; this treatment also extracts appreciable amounts of the reagent which is present in excess. It was found that the subsequent extraction of the ethyl acetate layer with dilute aqueous sodium carbonate produced a reddish brown artifact which passed into the aqueous phase and interfered seriously with the analytical procedure. Washing of the aqueous alkali layer with ethyl acetate removed this interfering coloration; it also removed some of the pyruvic acid hydrazone, giving rise to irregular calibration curves. Table I below shows the overall recovery of pyruvate when using ethyl acetate (washed carbonate layer), ether and chloroform. Clearly, chloroform gives the best results.

TABLE I

Efficiency of the extraction methods

Potassium pyruvate in mg	*Theoretical equivalent in PDH* mg/ml*	*O.D.***	*Measured O.D.*	*Per cent recovery*	*Extraction method*
3.69	7.84	1.144	0.682	59.6	Ethyl acetate (washed)
2.76	5.88	0.859	0.542	63.0	
1.84	3.92	0.573	0.381	66.5	
0.92	1.96	0.286	0.207	72.3	
3.00	6.38	0.930	0.720	77.5	Ether
2.25	4.78	0.700	0.428	61.1	
1.50	3.19	0.465	0.328	70.5	
0.75	1.59	0.231	0.195	84.5	
2.42	5.14	0.750	0.653	87.1	Chloroform
1.81	3.85	0.562	0.490	87.3	
1.21	2.57	0.375	0.326	86.9	
0.605	1.28	0.187	0.164	87.7	

* PDH, 2,4-dinitrophenyl hydrazone of pyruvic acid.

** Estimated from calibration curves using synthetic PDH repeatedly crystallised from ethylacetate (*trans* isomer only).

When estimating pyruvate in culture liquids by this method it was found that traces of other coloured compounds were also formed. The pyruvic acid hydrazone was separated from these by chromatographing in butanol–ammonia and eluting from the paper the slower spot of the two isomeric pyruvic 2,4-dinitrophenyl hydrazones (*trans* isomer[13]). The recovery in this step, determined with synthetic 2,4-dinitrophenyl hydrazone of pyruvic acid, repeatedly crystallised from ethyl acetate (pure *trans* isomer by chromatography), was 81 %.

Hence, the overall recovery of the method, including both the extraction and chromatographic steps, is 87 × 81 = 71 %. The concentration of pyruvic acid in the supernatant liquids was estimated from the amount of the *trans* isomer of the 2,4-dinitrophenyl hydrazone of pyruvic acid which was extracted and isolated by the above procedure, on the basis of a 71 % overall recovery.

Cell nitrogen: The amount of nitrogen contained in the cells was determined by the Kjeldahl method applied on a semi-micro scale. The cells contained in a known volume of culture were separated by centrifugation, suspended in sterile medium, re-centrifuged and then digested with sulphuric acid using mercuric oxide as catalyst. The ammonia formed was steam distilled over alkali, collected in dilute boric acid and titrated with hydrochloric acid.

Bacteriological

The strain of *A. vinelandii* used was the same as employed by previous workers in this laboratory[2,14-16] and was originally derived from the stock culture at the Technische Hoogeschool, Delft.

Medium: The organism was grown in a medium similar to the one used previously in this laboratory[2,14-16] the only difference being the addition of a small amount of citric acid (0.110 g/l) in order to increase the amount of iron in solution[17]. The slight precipitate formed during the preparation of the medium was removed by filtration. The pH of this medium was 7.08 as opposed to 7.26 to 7.28 for the same medium containing no citric acid.

Hydrazine containing medium was prepared by adding, under aseptic conditions, small volumes of hydrazine sulphate previously sterilised by autoclaving. Potentiometric titration of the medium showed that hydrazine sulphate behaved like a monobasic acid; consequently, an equivalent amount of sterile sodium hydroxide solution was also added in order to neutralise the effects of hydrazine sulphate on the pH of the medium.

Cell counts: The number of cells per unit volume of culture was determined in a standard Hawksley Thoma counting chamber.

Cultures: In order to provide efficient aeration, the cultures were grown in Kjeldahl flasks shaken on a microid shaker at 30°. The rate of shaking was such as to maintain a completely broken surface on the culture with the liquid splashing up to the first third of the neck of the flask. Flasks of capacity 250 and 50 ml and containing 100 and 20 ml of medium respectively, were used for growth experiments. Under these conditions of shaking, the cells remained entirely dispersed and no pellicles were formed.

Kinetic experiments

Preparation of cultures: In these experiments hydrazine was added to the culture in its logarithmic phase of growth. Inocula were prepared by inoculating sterile medium (20 ml) in a Kjeldahl flask (50 ml capacity) from an agar slope and shaking at 30° for 24 h. 1 ml of this culture was then transferred to another Kjeldahl flask containing 20 ml medium and shaken for another 24 h. 1 ml of this culture (population 1 to $2 \cdot 10^9$ cells/ml) was then transferred to 100 ml of sterile medium in a 250-ml Kjeldahl flask and shaken at 30°. After 17–22 h, depending on the strength of the culture desired, a small volume of sterile hydrazine sulphate solution (1 % of total) and the equivalent amount of sterile sodium hydroxide solution were added, and shaking was continued at 30°.

Disappearance of hydrazine: The rate of hydrazine disappearance was determined by withdrawing samples at time intervals, centrifuging and estimating the hydrazine content of the clear supernatant by the azine or picryl chloride method. When using the azine method the sample was made 1 *M* in hydrochloric acid immediately on withdrawing from the culture flask in order to avoid any losses due to aerial oxidation of the hydrazine. When using the picryl chloride method the sample was made 0.015 *M* in acetic acid. This reduced the pH to about 4.7, which is sufficiently acid to prevent the aerial oxidation of hydrazine. This small amount of acid did not affect the final pH of the coloured solution or the intensity of the colour.

Pyruvic acid determinations: In some kinetic experiments, pyruvic acid was estimated, as well as cell nitrogen, cell number, and both total and free hydrazine. In these experiments the volume of the sample was 20 ml and it was pipetted directly into dilute acetic acid (5 ml of 0.075 *M*). The suspension was centrifuged until clear and the supernatant decanted and used as follows: 20 ml were pipetted into 2,4-dinitrophenyl hydrazine reagent for the pyruvic acid estimation; 2 ml, after suitable dilution, were used for "free" and "total" hydrazine determination.

The cells remaining in the centrifuge tube were suspended in sterile medium

(5 ml) re-centrifuged and transferred into a micro Kjeldahl flask for cell nitrogen determination.

Reactions of supernatant liquids from hydrazine disappearance experiments

The cultures used in these experiments were prepared as for the kinetic experiments. A sterile solution of hydrazine sulphate (1 to 2 ml of $5 \cdot 10^{-2}$ *M*) was added to the culture 15–24 h after inoculation and the culture was shaken at 30° for a further 6–8 h. At the end of this time all the hydrazine had disappeared. The culture was then centrifuged and the clear supernatant decanted.

Reaction with picryl chloride: Supernatant liquid was mixed with half its volume of a 0.2 % alcoholic solution of picryl chloride, a small amount of solid sodium bicarbonate was added and the solution was allowed to stand overnight at room temperature. The almost opaque brown reaction mixture was then reduced to about one third of its volume by distilling under vacuum at 30 to 40°, made about 1 *M* with respect to hydrochloric acid, and extracted with chloroform. The yellow chloroform layer was in turn extracted with aqueous sodium bicarbonate (2 %, w/v) giving a dark brown solution which was acidified and extracted into a small volume of chloroform. This final extract, which contained all the reaction products, was investigated by paper chromatography using 0.5 *M* ammonium hydroxide and butanol–ammonia as solvents. The other layers, remaining after extraction, were found to contain only picryl chloride and its hydrolysis product, picric acid.

Reaction with 2,4-dinitrophenyl hydrazine: The supernatant liquid was mixed with one fourth of its volume of 0.01 *M* 2,4-dinitrophenyl hydrazine in 5 *M* hydrochloric acid and the solution allowed to stand at room temperature overnight. The reaction mixture was then extracted two or three times with ether and then with ethyl acetate until completely colourless. The combined ether layers were in turn extracted with 10 % aqueous sodium carbonate giving a reddish brown solution. The extracted ether layer, which was still yellow, and the ethyl acetate layer were examined by paper chromatography, after suitable concentration, and found to contain nothing but 2,4-dinitrophenyl hydrazine.

The reaction products were recovered from the aqueous carbonate layer by acidifying and extracting into ethyl acetate. Chromatograms of this final concentrate consisted almost entirely of the two isomeric hydrazones of pyruvic acid. Blank systems using water, 1.5 % aqueous mannitol, sterile medium and culture liquids from cultures to which no hydrazine was added, extracted in the above way, were found to contain a number of coloured spots. The intensity of these spots relative to that of the pyruvic acid hydrazones was very low, and they were hardly noticeable in chromatograms of culture liquids in which hydrazine had disappeared.

Reaction with picryl hydrazine: Culture liquids (25 ml) were reacted with a 0.14 % alcoholic solution of picryl hydrazine (15 ml) under acid conditions (2.5 ml of conc. HCl) at room temperature. After removing most of the ethanol by distilling *in vacuo* at room temperature, the reaction products, and some of the excess reagent, were extracted by shaking with chloroform. Extraction of this chloroform layer into aqueous alkali did not lead to a further separation as picryl hydrazine is also extracted.

The reaction products were identified as the picryl derivatives of pyruvic acid by comparison with similar extracts from reaction mixtures containing potassium pyruvate and picryl hydrazine, and with synthetic picryl hydrazone of pyruvic acid. This latter was prepared by dissolving powdered picryl hydrazine (50 mg) in one to two drops of anhydrous pyruvic acid and diluting with water (2 ml). The yellow crystalline precipitate was crystallised repeatedly from water. Picryl hydrazine was prepared from picryl chloride and hydrazine hydrate[18].

RESULTS

Growth in hydrazine containing media

Small inocula of *A. vinelandii* fail to multiply in media containing initial hydrazine concentrations greater than $7\text{–}9 \cdot 10^{-6}$ *M*. This inhibitory effect is reversible and, as the hydrazine concentration is reduced by aerial oxidation to non-toxic levels, growth appears in tubes which initially contained concentrations of up to 10^{-4} *M* in hydrazine. The presence of a supply of fixed nitrogen (5 mg of ammonium acetate nitrogen per 100 ml) does not counteract this inhibition.

In cultures which are aerated by vigorous shaking the rate of hydrazine disappearance due to aerial oxidation is not affected by the presence of small inocula (10^8 cells/100 ml). Appearance of growth is delayed until the concentration of hydrazine is reduced to a non-toxic level; thus, growth in media initially 10^{-4} and $1.5 \cdot 10^{-5}$ *M* was delayed by 30 and 12 h respectively. In the latter case growth was accompanied by a sharp drop in hydrazine concentration as can be seen from Table II.

TABLE II

DISAPPEARANCE OF HYDRAZINE FROM INOCULATED AND BLANK MEDIUM AT 30°

Solution A: Inoculated with 10^8 cells at time 0. Volume 100 ml. C_0, 0.0000153 *M* hydrazine.
Solution B: Blank. C_0, 0.0000132 *M* hydrazine.

Time (h)	*Solution A* C_t/C_0	*Solution B* C_t/C_0
0	1.00	1.00
3	0.87	0.84
6	0.85	0.82
9	0.79	0.77
12	0.77	0.72
26 "Initial"	0.05 After 17 h 0.62	0.37
33 "Initial"	0.06 After 17 h 0.64	0.25

Disappearance of hydrazine in dense cultures

By the azine method: By adding small amounts of hydrazine to cultures already in an advanced stage of growth (5 to 10 mg of cell nitrogen/100 ml) and by following the concentration of hydrazine in the supernatant by the azine method, rapid disappearance was observed. The half-life of the reaction depends on initial concentration (*cf.* curves A and B, Fig. 1) and on bacterial population (*cf.* curves B and C, Fig. 1).

The O.D. of every point in curves A, B and C was determined 15 min after adding the azine reagent. Normally this time is sufficient for the colour intensity to become constant. In this case, however, the O.D. of all points, except those for time 0, increased with time, reaching, in all cases, the same equilibrium value within 24–48 h. This quantitative reappearance of the colour could also be achieved by hydrolysing the sample with acid prior to adding the *p*-dimethylaminobenzaldehyde reagent. In this way, curve D, which is a composite from points from curves A, B and C, was obtained.

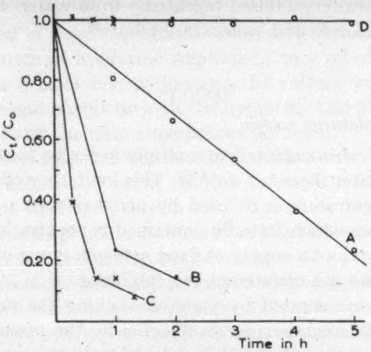

Fig. 1. Disappearance of hydrazine from cultures (by the azine method). A. $C_0 = 5.23 \cdot 10^{-4}$ *M*; No. of cells/ml, $7.2 \cdot 10^8$; B. $C_0 = 1.04 \cdot 10^{-4}$ *M*; No. of cells/ml, $8.0 \cdot 10^8$; C. $C_0 = 1.06 \cdot 10^{-4}$ *M*; No. of cells/ml, $1.2 \cdot 10^9$; D. Points from A, B and C after acid hydrolysis.

The above results suggest that the observed disappearance of hydrazine is due to the formation of a compound which stays in the supernatant liquids, and from which hydrazine can be obtained quantitatively by acid hydrolysis. It is also worth noting that the presence of the bacteria protects hydrazine from aerial oxidation. Thus, the concentration of total hydrazine (*i.e.* free plus acid labile) is not appreciably different from the initial concentration for periods of time during which, in blank experiments using sterile medium, a 20 to 30 % reduction was observed.

By the picryl chloride method: A typical disappearance curve in which hydrazine was estimated by the picryl chloride method is shown in Fig. 2a. The O.D. of each point was determined 5 min after adding the picryl chloride reagent, this time being sufficient for the full production of the violet hexanitrohydrazobenzene. The accompanying Fig. 2b shows the subsequent variation in each point with time. However, in this case, the increase in O.D. is not due to the slow liberation of hydrazine by hydrolysis but to the production of new coloured compounds through a slow reaction between picryl chloride and other substances accumulating in the supernatant liquids. This was proved by allowing samples from various parts of the curve to react with picryl chloride for 12 h, extracting the products in suitable solvents and examining them by paper chromatography. Points from the limiting part of the disappearance curve showed a brown and an orange spot but no violet hexanitrohydrazobenzene; the initial sample contained only hexanitrohydrazobenzene while the intermediate points contained progressively more of the "brown" and "orange" components.

Corrected curves: Clearly, because of the contribution to the total colour made by the brown and orange components, disappearance curves obtained by the picryl chloride method do not give a true picture of hydrazine disappearance. Such curves can be corrected by subtracting the limiting value from the rest of the curve by proportional redistribution. The justification for such a correction is that the increase in the O.D. of each point over an arbitrary period of time after the fifth minute (*i.e.* Δd_{5-15}), which is entirely due to the slow formation of the brown and orange components, varies inversely with the O.D. at the 5th min as shown below,

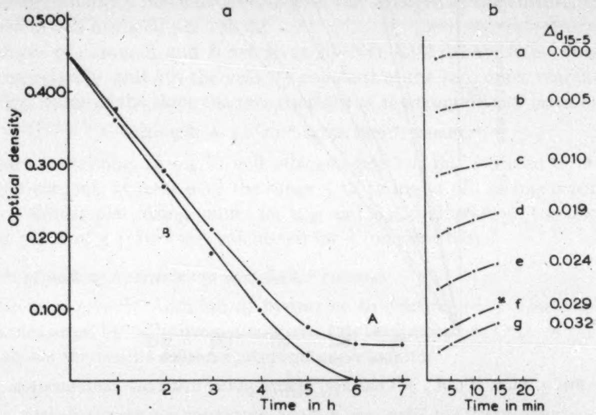

Fig. 2a. Disappearance of hydrazine by the picryl chloride method. Initial hydrazine concentration: $1.05 \cdot 10^{-3}$ *M*. Curve A. O.D. at the 5th min. Curve B. Corrected curve. Fig. 2b. Variation in O.D. with time. Reagent added at time 0.

O.D. at 5th min	*Δd_{5-15}*
0.450	0.00
0.380	0.005
0.290	0.010
0.210	0.019
0.130	0.024
0.080	0.029
0.050	0.032
0.050	0.032

It is reasonable to assume that the contribution due to these components at the 5th min (*i.e.* Δd_{0-5}) also follows the same relationship.

Curves corrected in this way are linear almost over the whole concentration range with a slope slightly greater than that of the uncorrected curves (*cf.* curve B, Fig. 2a).

Rate of hydrazine disappearance in relation to hydrazine concentration and bacterial population: The zero order reaction with respect to hydrazine concentration which is implied by the shape of the corrected curves was confirmed by measuring the initial rate of disappearance from cultures of identical strength at different hydrazine concentrations. As can be seen from Fig. 3, the time necessary to reduce the initial concentration by 5, 10 or 15 % is directly dependent on initial concentration. The linearity holds both for the corrected and uncorrected curves although the absolute rate is slightly different in the two cases. The cultures used in these experiments were of identical bacterial densities as they were obtained from the same master culture by subdivision.

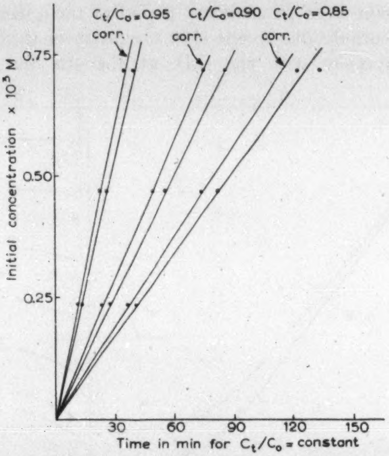

Fig. 3. Plots of C_t/C_0 = Constant, against initial hydrazine concentration.

The effect of bacterial density on the rate of hydrazine disappearance could not be demonstrated by such a direct experiment, as not only subdivision but volumetric dilution of the master culture with sterile medium would also be necessary. Such dilution led to lag periods and other irregularities which made the experiment impossible.

The relationship between rate of disappearance, initial hydrazine concentration and strength of culture is shown in Fig. 4. In curves A and B, $t_{0.50}$ and $t_{0.80}$ are plotted against C_0/N, where C_0 is the initial hydrazine concentration, and N is the nitrogen content, in mg, of the cells contained in 100 ml of culture.

The experimental points used for these curves were taken from Table III.

The relationship is linear, although the deviations shown by individual points are greater than those in Fig. 3. This is partly due to the additional experimental errors involved in estimating the nitrogen content of the cells, and partly because

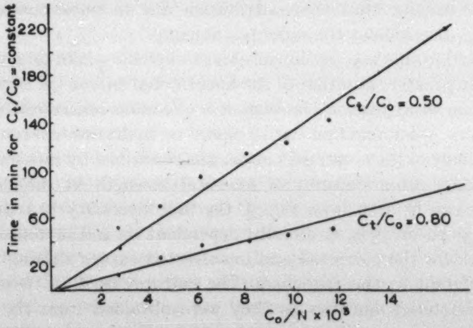

Fig. 4. Rate of hydrazine disappearance in relation to initial concentration and culture strength.

the results were obtained from experiments on individual cultures under, by necessity, slightly different experimental conditions. As the relationship between time t for C_t/C_0 constant, and C_0 is linear (*cf.* Fig. 3), it follows from curves A and B in Fig. 4 that the former quantity depends inversely on the strength of the culture, expressed as milligram of cell nitrogen per 100 ml.

The slopes of curves A and B are given by $N(1\text{-}K)/k$, where K is equal to 0.50 and 0.80 respectively, and k is the velocity constant of the zero order reaction. From the numerical value of the slope the rate constant of the reaction can be calculated as

$$k \text{ (Corrected)} = 4.0 \cdot 10^{-6} \text{ moles, litre}^{-1}\text{, minute}^{-1}$$

for a culture containing 10 mg of cell nitrogen per 100 ml; k varies directly with cell nitrogen content, at least over the range 5 to 12 mg of cell nitrogen per 100 ml.

From a similar plot, using values for $t_{0.50}$ and $t_{0.80}$ derived from the uncorrected curves, the value of $3.4 \cdot 10^{-6}$ was calculated for k (uncorrected).

Other effects of adding hydrazine to azotobacter cultures

Inhibition of growth: Addition of hydrazine to cultures of *A. vinelandii* arrests growth as measured by cell nitrogen content, total cell count or O.D. of the culture. Table IV shows the results from two typical experiments.

TABLE III

DISAPPEARANCE OF HYDRAZINE FROM *A. vinelandii* CULTURES USING THE PICRYL CHLORIDE METHOD

Culture	$C_0 \times 10^{-4}\ M$	*N mg/100 ml*	$C_0/N \times 10^{-4}$	$C_t/C_0 = 0.50$ *Time (min)*		$C_t/C_0 = 0.80$ *Time (min)*	
				Exptl.	*Corr.*	*Exptl.*	*Corr.*
A	2.26	8.15	0.28	40	37	20	15
B	4.74	10.4	0.45	81	71	33	27
C	7.50	11.8	0.63	108	96	45	39
D	10.6	12.7	0.83	127	115	51	42
E	7.37	7.50	0.99	150	123	54	47
F	10.50	8.85	1.18	161	143	57	51
G	9.95	5.96	1.67	246	219	99	84
H	21.3	7.35	2.90	420		Abnormal	

TABLE IV

EFFECT OF HYDRAZINE ON GROWTH OF CULTURE AND ON PYRUVIC ACID CONTENT OF THE SUPERNATANT

Hydrazine added at time 0.

Time (h)	*Cell N mg/100 ml*	*O.D.* of culture*	*No. of cells/ml*	*Hydrazine concentration (corr.)*	*Pyruvic acid concentration*
Experiment 1					
0	7.40	0.430	$4.9 \cdot 10^8$	$7.37 \cdot 10^{-4}\ M$	0.00
2	7.50	0.470	$5.6 \cdot 10^8$	$3.95 \cdot 10^{-4}\ M$	$1.08 \cdot 10^{-4}\ M$
4	7.40	0.465	—	$1.64 \cdot 10^{-4}\ M$	$1.02 \cdot 10^{-4}\ M$
6	7.55	0.450	$4.6 \cdot 10^8$	$0.26 \cdot 10^{-4}\ M$	$0.93 \cdot 10^{-4}\ M$
Experiment 2					
0	6.00	0.393	—	$9.95 \cdot 10^{-4}\ M$	0.00
2	—	0.400	$3.6 \cdot 10^8$	$6.90 \cdot 10^{-4}\ M$	$0.81 \cdot 10^{-4}\ M$
4	6.20	0.397	$4.1 \cdot 10^8$	$4.65 \cdot 10^{-4}\ M$	$0.73 \cdot 10^{-4}\ M$
6	6.00	0.400	$3.3 \cdot 10^8$	$1.87 \cdot 10^{-4}\ M$	$0.88 \cdot 10^{-4}\ M$

* O.D. measured using 1-cm cells and a culture dilution of 4/5.

Growth, as measured by any of the above properties, is resumed after complete disappearance of hydrazine.

Production of coloured picryl derivatives: Supernatant liquids containing sequestered hydrazine react with picryl chloride to give dark brown solutions. Suitable extracts from such reaction mixtures were investigated chromatographically and found to contain the following spots:

Spot	*R_F value*			*Colour*	
	*Solvent**				
	1	*2*	*3*	*Acid*	*Alkali*
a	0	0	0.96	Yellow	Orange
b	0.45	0.57	0.45	Yellow	Violet
c	0.55	0.66	0.88	Yellow	Brown
d	0.71	0.77	0.72	Yellow	Yellow

* Solvent 1: *n*-butanol–0.5 *M* ammonium hydroxide. Solvent 2: *n*-butanol–ethanol–0.5 *M* ammonium hydroxide (70:10:20). Solvent 3: 0.5 *M* ammonium hydroxide.

Of these spots b was identified as hexanitrohydrazobenzene by direct comparison with the reaction product of hydrazine and picryl chloride. This spot was only found in experiments in which hydrazine had not disappeared completely. Spot d was due to picric acid which is formed by the hydrolysis of picryl chloride.

Spots a and c are produced as a direct result of hydrazine disappearance and they are not found in blanks carried out with sterile medium, growth supernatant or supernatant to which hydrazine was added after the removal of bacteria by centrifugation.

If supernatant liquids containing sequestered hydrazine are hydrolysed with acid prior to reacting with picryl chloride, then no trace of spots a and c is found, the chromatograms consisting entirely of picric acid and hexanitrohydrazobenzene. This fact, which confirms the "reappearance" of hydrazine on acid hydrolysis observed by the azine method, also shows that the substances, which react with picryl chloride to give spots a and c are completely destroyed by acid.

Of the two coloured picryl derivatives, the orange decomposes slowly in the weakly alkaline chromatographic solvent. The brown, however, being more stable, can be eluted from the chromatograms and estimated colorimetrically; similarly, by eluting the violet hexanitrohydrazobenzene, an estimate can be made of the concentration of free hydrazine in the culture liquids. The values given in Table V below, obtained in this way, show that the concentration of the brown compound increases with decreasing concentration of free hydrazine.

TABLE V

Hydrazine added at time 0.

Time (h)	*O.D. of eluted hexanitro-hydrazobenzene spot × ¼*	*O.D. of eluted brown spot*
0	0.410	0.000
2	0.220	0.080
4	0.145	0.230
6	0.025	0.420

In the same way, using cultures in which hydrazine had disappeared completely, it was found that the concentration of the brown component was independent of the density of the bacterial culture for a given initial hydrazine concentration, but that it varied, almost linearly, with initial hydrazine concentration.

Production of pyruvic acid: Supernatant liquids containing sequestered hydrazine were treated with 2,4-dinitrophenyl hydrazine under acid conditions and the products were extracted and investigated by paper chromatography using the standard procedures[11, 12]. The original ethyl acetate layer, after extraction with sodium carbonate, contained only excess reagent while the sodium carbonate layer, on paper chromatography, was found to contain the two isomeric hydrazones of pyruvic acid. That the production of pyruvic acid is a direct result of hydrazine disappearance can be seen from Table VI below, which shows the O.D. of the carbonate layers, obtained under identical conditions, from the reaction mixtures of various blank systems with 2,4-dinitrophenyl hydrazine.

TABLE VI

Reaction: 2,4-dinitrophenyl hydrazine with		*O.D. (blue filter)*
1. Supernatant containing sequestered hydrazine		2.5·0.750
2. Supernatant containing no hydrazine	a	0.250
	b	0.248
3. Sterile medium	a	0.102
	b	0.125
4. 1.5% aqueous mannitol		0.070
5. Distilled water		0.068

The presence of pyruvic acid was confirmed by treating supernatant liquid containing sequestered hydrazine with an alcoholic solution of picryl hydrazine under acid conditions. Chloroform extracts of such reaction mixtures were investigated by paper chromatography and found to contain the following three spots,

Spot	*R_F value*		*Colour*			*Substance*
	*Solvent 3**	*Solvent 2**	*On paper (wet)*	*On paper (dry)*	*In NH_3 vapour*	
A	0.65	0.40	Orange	Yellow	Pink	Picryl hydrazine
B	0.76	0.60**	Brownish	Yellow	Brown	Due to pyruvic acid
C	0.85	0.20**	Brown	Yellow	Brown	Due to pyruvic acid

* Solvents as described for the chromatography of the picryl derivatives.
** These R_F values are approximate as substance is unstable in the chromatographic solvent and only short runs were possible.

Identical spots were obtained from chromatograms of synthetic pyruvic picryl hydrazone, or from the reaction mixture of pyruvic acid and picryl hydrazine treated under the same conditions as used when investigating the culture liquids.

Spot "B" decomposes slowly in solvent 2 to give some residue, which stays at the origin, and a long hydrolysis trail which contains spots A and C. The fact that chromatograms of pyruvic picryl hydrazone, purified by repeated crystallizations from water, still contain picryl hydrazine, suggests that the latter is formed by the decomposition of the hydrazone.

It is significant that none of the reaction products with picryl hydrazine is identical with the "brown" or "orange" components observed in the picryl chloride reaction.

Production of pyruvic acid in relation to sequestered hydrazine and the picryl derivatives: Supernatant liquid containing sequestered hydrazine was treated with picryl chloride, and the brown and orange products were removed from the acidified reaction mixture by extracting with ethyl acetate. The remaining aqueous layer, on reacting with 2,4-dinitrophenyl hydrazine, was found to contain pyruvic acid.

Essentially the same experiment was repeated quantitatively. Three aliquots, A, B and C, of supernatant containing sequestered hydrazine were used. A was treated with picryl chloride and extracted with ethyl acetate until colourless. B was treated in exactly the same way but without using any picryl chloride, the purpose of this blank being to determine the amount of pyruvic acid lost into the ethyl acetate during the extraction of the coloured compounds. The extracted aqueous phases of A and B together with C (which was a normal aliquot, not treated in any way) were reacted with 2,4-dinitrophenyl hydrazine. By extracting the products, chromatographing, eluting the two isomeric hydrazones and determining their O.D. the following results were obtained:

Pyruvic hydrazone spots (solvent 2)	*Optical density of elute*		
	A	*B*	*C*
"Slow" (*trans*)	0.220	0.235	0.525
"Fast" (*cis*)	0.060	0.100	0.208

Clearly, solutions A and B contain substantially the same amount of pyruvic 2,4-dinitrophenyl hydrazone, but less than C, which was not submitted to an extraction process.

Using this method for determining the 2,4-dinitrophenyl hydrazone of pyruvic acid, the relationship between pyruvic acid and sequestered hydrazine concentration was investigated. As can be seen from Table IV, above, the production of pyruvic acid in cultures to which hydrazine is added, shows no relation to the hydrazine disappearance curve. The concentration of pyruvic acid is more or less constant after

TABLE VII

All concentrations measured 2 h after addition of hydrazine.

Culture	*Pyruvic acid* $\times 10^{-4}$ *M*	*Hydrazine concentration* $\times 10^{-4}$ *M*			*Cell nitrogen mg/100 ml*
		C_0	C_t	C_0–C_t	
1	1.08	7.37	4.00	3.37	7.4
2	0.81	9.95	6.93	3.02	6.0
3	0.81	7.50	2.85	4.65	11.9
4*	0.84	10.6	3.70	6.36	12.7

* Concentrations determined 2.5 h after hydrazine addition.

the second hour, and is at all times very small by comparison to the total, or sequestered hydrazine concentration.

From experiments carried out on different cultures no relation could be found between the amount of pyruvic acid formed and initial (C_0), free (C_t), or sequestered (C_0–C_t) hydrazine concentration; nor did the strength of the culture appear to have any significant effect on the pyruvic acid level, as can be seen from Table VII.

DISCUSSION

From the experimental evidence presented above it is clear that the observed hydrazine disappearance is in effect a detoxification process by which hydrazine is converted into an acid labile compound which accumulates outside the cells. The nature of this compound has not been elucidated, but the ease with which it liberates hydrazine on acid hydrolysis suggests that it contains hydrazine bound to an organic residue by a hydrazone ($R_1R_2C = NNH_2$) or hydrazide ($RCONHNH_2$) linkage. As hydrazine can react at both ends, ring formation is also possible; in such a case, a cyclic azine, hydrazide, or hydrazone-hydrazide may be formed.

Supernatant liquids containing bound hydrazine also contain some other compound or compounds which can react with picryl chloride to yield coloured picryl derivatives. The ability of these compounds to react with picryl chloride is destroyed under the acid conditions which liberate hydrazine from its bound form. Recently, HARRIS[19] has demonstrated that the converse is also true, *i.e.* formation of the picryl derivatives precludes liberation of hydrazine by acid hydrolysis. Moreover, the colour intensity of one of the picryl derivatives (brown) is directly proportional to the concentration of acid labile hydrazine. The above suggests that these compounds and "bound" hydrazine are identical, and that the coloured picryl compounds are in fact the picryl derivatives of sequestered hydrazine.

The accumulation of pyruvic acid in the surrounding medium on adding hydrazine to *A. vinelandii* cultures appears to be incidental to the phenomenon of hydrazine disappearance. The lack of stoichiometry between the concentration of pyruvic acid and acid labile hydrazine, and the simultaneous isolation of both pyruvic acid and the coloured picryl derivatives, show that pyruvic acid is not the organic acceptor responsible for binding hydrazine.

Failure to isolate any carbonyl compounds other than pyruvic acid, under conditions which liberate hydrazine, argues against the hydrazine being bound in a hydrazone or azine type of compound. Chromatographic investigation of synthetic N-acetyl picryl hydrazine[8], N-benzoyl picryl hydrazine[19] and the reaction product of α-glutamyl hydrazide with picryl chloride reveals two spots, one "orange" and one "brown" with R_F values similar to those of the picryl derivatives of sequestered hydrazine. The two coloured compounds are always present and appear to be either isomers, or else directly related to each other. The similarity between the chromatograms of the picryl derivatives of sequestered hydrazine and those of the three synthetic picryl hydrazides suggests that hydrazine disappears by combining with an acid residue to give a hydrazide.

The kinetic characteristics of the disappearance reaction, *i.e.* first order dependence on cell substance, and zero order with regard to hydrazine concentration, are consistent with an enzymic reaction in which hydrazine is used as a substrate.

These two conditions can also be satisfied by a scheme in which hydrazine reacts chemically with the products of an enzymic reaction of the cell,

$$\text{Enzyme} \longrightarrow \text{X} + \text{NH}_2\text{–NH}_2 \longrightarrow \text{sequestered hydrazine,}$$

provided that the chemical reaction is fast and that the enzymic reaction leading to the formation of X is rate determining. These assumptions also imply that the system is in a "steady state" and that the concentration of "X" is much lower than that of free hydrazine.

It may be that the phenomenon of disappearance is directly responsible for the observed inhibition of growth in the presence of hydrazine. In such a case, inhibition could be due to competition of hydrazine for a metabolite vital to the growth of the organism. It is interesting to note that HARRIS[19] has found that ammonia can remove at least some of the inhibitory effects of hydrazine and at the same time reduce the rate at which hydrazine disappears from such systems. Competition of hydrazine with ammonia in enzymic amidation[20, 21] reactions, and reactions involving imine formation is well known.

BACH[3] found that hydrazine disappeared from heavy suspensions of resting *Azotobacter* cells by combining with α-ketoglutaric acid to form 3,4-dihydropyrazinone-5-carboxylic acid (PCA) and other condensation products. However, the disappearance observed in our case must proceed by a different mechanism as no 2,4-dinitrophenyl hydrazone of α-ketoglutaric acid could be isolated from the acid hydrolysate of culture liquids containing sequestered hydrazine. Moreover, solutions of PCA, or solutions containing equimolar amounts of α-ketoglutaric acid and hydrazine, on treating with picryl chloride under the same conditions as used for the formation of the picryl derivatives of sequestered hydrazine gave no trace of the "brown" or "orange" compounds.

This difference in the mechanism of the disappearance reaction may be due to the fact that in BACH's experiments no outside supply of carbohydrate was available to the cells, whilst in our case mannitol was present, under conditions which inhibit the respiration of the culture only partially[2]. Clearly, in cultures in which oxidative glycolysis is taking place, organic acceptors (or the energy necessary for an enzymic reaction) may be available for the binding of hydrazine, which are not present in the resting systems used in BACH's experiments. That this may be the case is also suggested by the fact that the amount of hydrazine bound, over comparable periods of time, in relation to the quantity of the cell substance present, is much higher in our case (10^{-5} moles of hydrazine/mg of cell nitrogen as opposed to $3 \cdot 10^{-8}$ moles/mg of N in BACH's experiments).

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REFERENCES

[1] D. BURK AND C. K. HORNER, *Naturwissenschaften*, 23 (1935) 259.
[2] M. A. AZIM AND E. R. ROBERTS, *Biochim. Biophys. Acta*, 21 (1956) 562.
[3] M. K. BACH, *Biochim. Biophys. Acta*, 26 (1957) 104.
[4] E. D. ROSENBLUM AND P. W. WILSON, *J. Bacteriol.*, 61 (1951) 475.

[5] M. A. Azim, *Ph.D. Thesis*, University of London, 1953.
[6] M. Pesez and A. Petit, *Bull. soc. chim. France*, (1947) 122.
[7] G. W. Walt and J. D. Chrisp, *Anal. Chem.*, 24 (1952) 2006.
[8] H. Leemann and E. Grandmougin, *Berichte*, 41 (1908) 1295.
[9] L. M. Kul'berg and A. I. Cherkessov, *Zhur. Anal. Khim.*, 6 (1951) 364.
[10] J. P. Riley, *Analyst*, 79 (1954) 76.
[11] D. Cavallini, N. Frontali and G. Toschi, *Nature*, 163 (1949) 368.
[12] M. F. S. El Hawary and R. H. S. Thompson, *Biochem. J.*, 58 (1953) 340.
[13] H. B. Stewart, *Biochem. J.*, 55 (1953) xxvi.
[14] B. A. Pethica, E. R. Roberts and E. R. S. Winter, *Biochim. Biophys. Acta*, 14 (1954) 85.
[15] T. G. G. Wilson and E. R. Roberts, *Biochim. Biophys. Acta*, 15 (1954) 390.
[16] J. A. Rakestraw and E. R. Roberts, *Biochim. Biophys. Acta*, 24 (1957) 555.
[17] D. Burk, *Soil Sci.*, 33 (1932) 413.
[18] W. Borsche, *Ber.*, 54 (1921) 1287.
[19] P. Harris, private communication.
[20] J. F. Speck, *J. Biol. Chem.*, 179 (1949) 1387.
[21] M. Schou, N. Grossowitz and H. Waelsch, *J. Biol. Chem.*, 192 (1951) 187.

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SOME PHYSICAL PROPERTIES OF DEOXYRIBONUCLEIC ACIDS DISSOLVED IN A HIGH-SALT MEDIUM: SALT HYPERCHROMICITY

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SUMMARY

1. The effects of very high salt concentrations upon the u.v. absorption, viscosity, and optical rotation of DNA were studied.

2. Concentrated NaBr (7.4 *M*) caused a pronounced hyperchromicity (measured at 260 mμ) which was entirely reversible when excess salt was removed. The increased absorption (salt hyperchromicity) was approximately linear with salt concentration.

3. Heating of a DNA solution in saturated NaBr produced a further increase in absorption which was irreversible and independent of DNA concentration.

4. Salt hyperchromicity, which was most pronounced in NaBr solutions, was less evident in other halide and alkali salts, and completely absent with LiCl solutions.

5. Gradually increasing NaBr concentration produced a loss in dextrorotation of DNA from an initial value of about + 117 degrees to a final value of + 67 degrees at 7.4 *M* salt concentration. The rate of change in specific optical rotation was most pronounced in 4 to 5 *M* NaBr concentrations.

6. Concentrated NaBr solutions of DNA showed a striking loss in viscosity as compared with DNA solutions in 0.1 *M* salt.

7. Neutral salt concentration in excess of about 13 molal caused quantitative precipitation of DNA. Among the salts which were tested, precipitation of DNA resulted only from the use of LiCl solutions.

8. Some structural implications of these phenomena were discussed, and an attempt was made to compare the various types of hyperchromicity ("hydrolytic", "water", and "salt") in nucleic acids.

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INTRODUCTION

It has been observed repeatedly[1–4] that a solution of deoxyribonucleic acid (DNA) in distilled water is hyperchromic in comparison with a solution of DNA in dilute salt. Although the magnitude of this hyperchromicity can exceed a value of 17 % (measured at the absorption peak of nucleic acids—260 mμ), a detailed description of the mechanics of this considerable increase in absorption is lacking. Furthermore, the phenomenon is complicated by the fact that the addition of salt to a solution of DNA in distilled water does not completely eliminate the hyperchromicity. Such a DNA sample which has been exposed to distilled water is said to be "partially denatured by dilution"[2].

A recent report from this laboratory[5] mentioned another form of hyperchromicity which was reversible. This new phenomenon occurred when the DNA was exposed to high-salt concentrations ($> 3\ M$). The present report attempts to relate these hyperchromic phenomena and to explore the effects of high-salt concentrations upon DNA in terms of u.v. absorption, viscosity, and optical rotation.

METHODS

Spectrophotometric, viscometric, and optical rotation techniques used in this study have been described[5]. All absorbancy and polarimetric measurements were made against the appropriate solvent blanks. Optical rotation measurements were carried out with the sodium illumination (589 mμ). The nucleic acid samples were isolated from spleen, kidney, and cerebellum of cattle[5,6]. These DNA samples had never been dialyzed or dried, nor exposed to ether, absolute alcohol, acids, or bases.

EXPERIMENTAL RESULTS

The hyperchromic effect of a high-salt concentration

Fig. 1 shows the relative changes in absorption spectra of DNA dissolved in various concentrations of salt. The dependency of absorption (at 260 mμ) upon the salt concentration is also evident from Fig. 2, in which it is shown that hyperchromicity begins to appear at 2 M salt concentrations, and reaches its maximum height in saturated (7.4 M) NaBr. Since the effect is also shown by NaCl and KCl, though to a lesser extent, it would seem that the phenomenon of salt hyperchromicity is due to other properties in addition to ionic strength. Further evidence that this hyperchromic effect was not caused by ionic strength alone was provided by LiCl. In this case, the absorption at 260 mμ was independent of salt concentration for any value between 1 and 6 M LiCl.

Whether hyperchromicity is correlated with a particular anion could not be decided with certainty since fluoride salts are too insoluble and iodide salts absorb so strongly in the u.v., that their use was precluded.

Reversibility of the hyperchromic effect

In Fig. 3, the spectra of two DNA samples dissolved in 0.1 M NaBr are compared. The sample portrayed in Curve A was exposed to 7 M NaBr for 30 min at room temperature, and then diluted to a given volume with 0.1 M NaBr. Curve B is the spectrum of a sample (dissolved in 0.1 *M* NaBr) which had not been exposed to salt concentrations greater than 1 *M*. Curve C shows the spectrum of DNA dissolved in 7 *M* NaBr. Thus, the identity of Curves A and B (in comparison to Curve C) proves the reversibility of salt hyperchromicity.

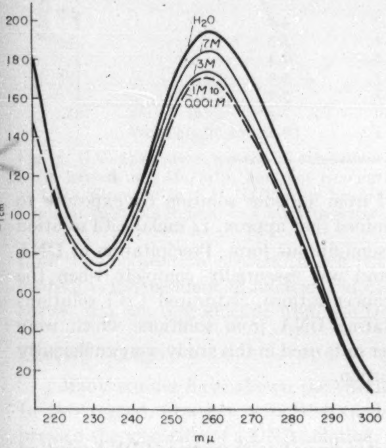

Fig. 1. U.V. absorption spectra of cattle kidney DNA as measured in various molar concentrations of NaBr.

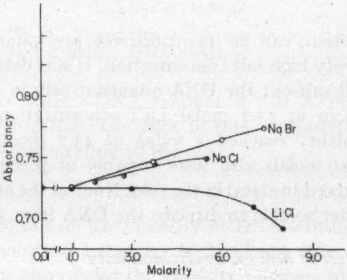

Fig. 2. Hyperchromicity of spleen DNA dissolved in various concentrations of alkali halide salts.

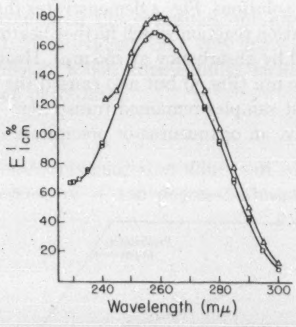

Fig. 3. U.V. absorption spectra of spleen DNA: reversibility of the salt hyperchromic effect. Spectra were measured in 7 *M*, △—△, Curve C, and in 0.1 *M*, ○—○, Curve B NaBr. A third spectrum was measured in 0.1 *M* NaBr after the concentrated DNA solution had been exposed to 7 *M* NaBr for 35 min at 25°, □—□, Curve A.

The salting-out of DNA from fourteen molal salt solution

Table I shows the approximate molarity and molality of saturated solutions of various halide salts used in this study. It was of great interest to find that DNA, like

TABLE I

MOLARITY AND MOLALITY OF HALIDE SALTS IN AQUEOUS, SATURATED SOLUTION AT 20°

Molarity, g/l; molality, g/1000 g of solvent.

Salt	*Molality*	*Molarity*
KCl	4.7	4.1
NaCl	6.2	5.5
NaBr	9.2	7.4
RbBr	6.3	5.0
RbCl	7.2	5.7
CsCl	10.6	7.2
LiCl	18.5	10.4

protein, can be quantitatively precipitated from aqueous solution by exposure to a very high salt concentration. It was determined that approx. 14 molal LiCl solution will salt-out the DNA quantitatively in a semi-fibrous form. Precipitation of DNA began at 13.2 molal LiCl concentration and was essentially complete when the molality reached a value of 13.7 (final concentration). Saturated CsCl solutions (10.6 molal) also were capable of precipitating DNA from solutions which were allowed to stand in the cold. None of the other salts used in this study were sufficiently water soluble to displace the DNA from solution.

Effect of heat on hyperchromicity

It was mentioned previously[5] that long standing of a concentrated DNA solution in 7 *M* NaBr at room temperature sometimes resulted in a flocculent precipitate or the development of a slight opaqueness of the solution. Since a similar degradation process also takes place in the cold (but more slowly), it was of interest to determine the effects of heating on such solutions. Fig. 4 demonstrates that heat (100°) produces an almost immediate degradation reaction which further heating for as long as 20 min does not change (as measured by absorbancy at 260 mμ). Heating not only caused an increase in absorbancy at 260 mμ (Fig. 5) but also caused the nucleic acid to become very sticky and viscous. Most samples remained transparent under these conditions of limited heating; eventually, an opaqueness or precipitate developed. Fig 6 shows

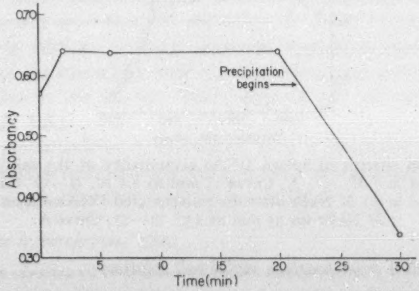

Fig. 4. Relationship between the duration of heating a solution of kidney DNA in saturated NaBr and the resultant absorbancy at 260 mμ.

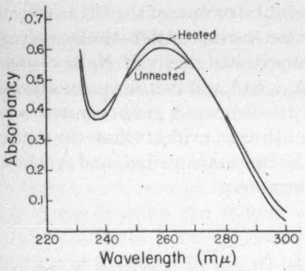

Fig. 5. U.V. absorption spectra of cerebellum DNA heated in 7 M NaBr. Samples (100 μg) of DNA dissolved in 7 M NaBr were heated 5 min at 100° and diluted to 5 ml with water.

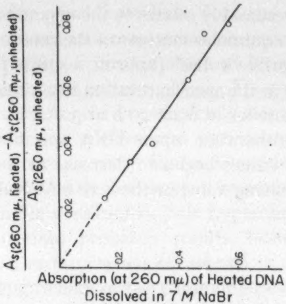

Fig. 6. Absorption increment at 260 mμ of heated kidney DNA dissolved in 7 M NaBr.

that the degradation process (which results when a DNA solution in 7 M NaBr is heated) is independent of nucleic acid concentration. The slope of this "degradation curve" was not reproducible from one DNA preparation to the next.

Viscosity changes

Many studies have shown the effects of salt on the viscosity of DNA solutions. In this regard, CONWAY AND BUTLER demonstrated that 0.1 M NaCl not only depressed the viscosity of a DNA solution, but also caused the viscosity numbers to be nearly independent of DNA concentration[7]. These authors also pointed out that the concentration-dependent curve for the viscosity of DNA in water, when extrapolated to zero concentration of DNA, would yield viscosity numbers similar to those obtained for DNA dissolved in 0.1 M salt.

In Fig. 7 I have compared the viscosities obtained in 7 M NaBr with those obtained in dilute KCl. It is evident that very high salt concentrations caused a marked viscosity depression which, incidentally, facilitated the filtration of DNA solutions during isolation procedures.

Optical rotation

Earlier investigations[8,5] revealed that dilute salt solutions of nucleic acids have positive rotations in excess of + 100 degrees. Almost the whole of this positive

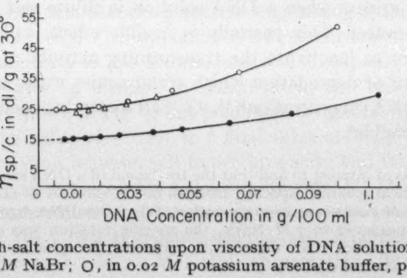

Fig. 7. Effect of high-salt concentrations upon viscosity of DNA solutions. ●, cattle cerebellum DNA dissolved in 7 M NaBr; ○, in 0.02 M potassium arsenate buffer, pH 7.0; △, in 0.1 M KCl.

value presumably relates to the uncompensated helical structure of the DNA molecule since an equimolar mixture of the constituent four nucleotides arithmetically averages to a negative value[*] (approx. —38°). Fig. 8 demonstrates that 7 *M* NaBr causes a decrease in the specific rotation to a value of about + 65°, and that the value attained is constant for at least 20 h at 30°. Fig. 9 shows the effects of a gradually increasing salt concentration upon DNA specific rotation. It was evident that the optical rotation values began to decrease at the 0.1 *M* NaBr concentration, and reached a lower limiting value in the 4 to 6 *M* NaBr concentrations.

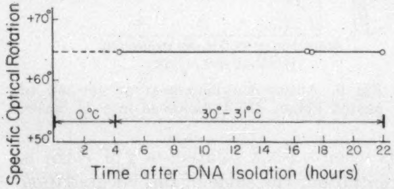

Fig. 8. Effect of time upon specific optical rotation of spleen DNA dissolved in 7 *M* NaBr.

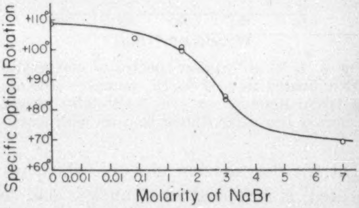

Fig. 9. Effect of salt concentration upon the optical rotation of spleen DNA.

DISCUSSION

The advantages which arise from the use of saturated sodium bromide in preference to molar sodium chloride for DNA isolation were discussed in an earlier publication[5]. Unfortunately, we know very little about the effects of a very high salt concentration upon the integrity of the DNA molecule. In the present study, the observed spectrophotometric, viscometric, and polarimetric changes caused by saturated NaBr were found to be reversible and, therefore, within the limits of these procedures, high-salt concentrations do not have irreversible degradative effects.

The protective effect of dilute salt upon DNA structure as well as the hyperchromic reaction of DNA exposed to distilled water have been widely studied. The present investigation of the effects of a high-salt concentration demonstrated that another hyperchromic shift appeared when the molarity exceeded a value of two. Thus, the hyperchromic phenomena of nucleic acids can be classified under three headings: (a) Hyperchromicity arising from degradation procedures (*e.g.*, acid, alkali, enzymes; this type (referred to here as "hydrolytic" hyperchromicity) is irreversible. (b) Hyperchromicity arising when a DNA solution in dilute salt is transferred (by dialysis) to distilled water. This partially reversible effect, called "water hyperchromicity", is known to inactivate the transforming activity of microbial nucleic acids[10], and the extent of degradation which accompanies water hyperchromicity is dependent upon the DNA concentration[2,11]. (c) "Salt hyperchromicity", which appears to be completely reversible[5].

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[*] In this regard, it was of interest to find that the treatment of a DNA solution with pancreatic nuclease reduced the optical rotation (specific) from an initial value of + 110° to a final value of + 12° (measured in 0.08 *M* potassium arsenate buffer). When this DNA digest, known to contain oligo-nucleotides[9], was measured in 7 *M* NaBr, the specific rotation was depressed further to + 7.9°. Treatment of the initial DNA digest with intestinal phosphatase reduced the specific rotation (in 0.08 *M* arsenate buffer) to + 5°.

These three expressions of hyperchromicity provide evidence that the complex structure of the DNA molecule can become altered in a manner which permits each purine and pyrimidine chromophore to contribute a greater share of its potential absorptive capacity to the chromophore system of the entire molecule. The maximum hyperchromic effect is produced when the nucleic acid is completely degraded by hydrolysis to the four constituent nucleotides (or purines and pyrimidines); this maximum increase in absorption can be as great as 40 %. Water hyperchromicity produces a 17 % increase in absorption and involves the breakage of hydrogen bonds only (some of which can re-form under suitable conditions). Salt hyperchromicity, which constitutes a 12 % increase in absorption, probably results, from limited cleavage of hydrogen bonds, all of which can re-form when excess salt is removed.

Application of the general rule[12], that lengthening of the chromophore enhances the total absorbancy of a system, leads to the prediction that, in the "native" DNA molecule, the chromophore interaction must be of a type that reduces the individual contribution of each heterocyclic chromophore. Present chemical knowledge of DNA structure indicates that there can be no interaction of the chromophores through covalent conjugation (*i.e.*, through the sugar-phosphate diester bonds). Thus, we are led to believe that the close proximity of the heterocyclic bases, stacked one above the other, results in an extension of the electron orbitals over more than one nucleotide[13]. Normally, this interaction must be such that each chromophore is unable to contribute its full capacity in the absorption of u.v. light—perhaps due to the fact that each nucleotide is slightly displaced along the helix (see ref. 14, p. 462), and does not lie directly over or under its neighbouring nucleotides. An alternative explanation is that these losses in absorption may be the result of shielding (see ref. 14, p. 488).

However, if the DNA helix could be forced to expand lengthwise, there would be less inhibitory action between the chromophores because of the greater internucleotide distance, and each base would be able to contribute a larger share to the total absorption. This possibility for explaining water hyperchromicity is supported by ROWAN's light scattering and flow birefringence studies wherein he showed that the DNA molecule lengthens as much as 38 % when transferred from dilute salt solution to pure water solution[15]. This lengthening is generally assumed to result from internucleotide charge repulsion. MICHELSON's data[13] demonstrate that even certain dinucleoside phosphates exhibit a marked hyperchromicity upon hydrolysis. Again, this indicates that the hyperchromic phenomena must result primarily form chromophore interaction between adjacent heterocyclic bases rather than through interaction of the chromophores in adjacent chains of the DNA double helix.

The experimental fact that the extensive lengthening of the DNA helix (as occurs in distilled water solutions) is accompanied by a hyperchromic shift leads me to suspect that salt hyperchromicity is also a consequence of an expansion of the molecule. An expansion necessitates an increase in helical pitch, and thereby, a loss in specific dextrorotation. As shown in Fig. 9, this prediction is fulfilled (specific optical rotation falls from an initial value of + 117° to a final value of + 67°). However, this interpretation of the relation between salt hyperchromicity and the decrease in dextrorotation is open to question upon the following grounds: (a) The decrease in dextrorotation is not a linear function of salt concentration—the maximum rate of change being observed in the 3 to 4 *M* region—whereas salt hyperchromicity increases in a linear fashion between 1 and 7.4 *M* NaBr concentration. (b) A decrease in optical rotation would also be expected when the DNA is transferred from dilute salt to distilled water. Such a decrease was not observed (Fig. 9).

It must be admitted that the manner in which a high-salt environment could cause a reversible extension of the helix is not clear. This problem is further complicated by the fact that different neutral salts produce different degrees of hyperchromicity and, in the case of LiCl, no trace of salt hyperchromicity was observed. Also, the effect does not appear to be correlated with the qualitative nature of either cation or anion. Among the salts tested, sodium bromide produced the most striking hyperchromicity.

When the DNA solution in saturated NaBr was heated to 100°, an almost immediate irreversible denaturation occurred. The DNA became very viscous, the hyperchromicity increased even more, and the DNA sometimes precipitated. These effects probably were the result of separation of the DNA double helices followed by collapse to a disoriented structure[16].

One of the important advantages arising from the use of saturated NaBr (9.2 molal) for DNA isolation results from the salting-out and removal of protein, including nucleases, from the DNA preparation[5]. However, exposure of DNA to neutral salt concentrations in excess of about 13 molal caused salting-out of the DNA itself. Among the salts tested, this effect was produced only with LiCl (at room temperature). Precipitation of DNA in this manner was probably the result of molecular aggregation which followed the removal of the water sheaths (ice structure) surrounding the DNA helices[17]. The potential usefulness of this precipitation method for separation of ribonucleic acid from DNA, for DNA isolation and fractionation, is now being investigated.

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REFERENCES

1. E. R. Blout and A. Asadourian, *Biochim. Biophys. Acta*, 13 (1954) 161.
2. R. Thomas, *Biochim. Biophys. Acta*, 14 (1954) 231.
3. J. Shack, R. J. Jenkins and J. M. Thompsett, *J. Biol. Chem.*, 198 (1952) 85.
4. C. F. Emanuel and I. L. Chaikoff, unpublished studies on deoxyribonucleic acids of normal and malignant tissues, 1953.
5. C. F. Emanuel and I. L. Chaikoff, *J. Neurochem.*, accepted for publication.
6. C. F. Emanuel and I. L. Chaikoff, *Biochim. Biophys. Acta*, 24 (1957) 254.
7. B. E. Conway and J. A. V. Butler, *J. Polymer Sci.*, 12 (1954) 199.
8. C. Tamm, M. E. Hodes and E. Chargaff, *J. Biol. Chem.*, 195 (1952) 49.
9. A. H. Gordon and P. Reichard, *Biochem. J.*, 48 (1951) 569.
10. S. Zamenhof, *Progress in Biophysics and Biophysical Chemistry*, Vol. 6, Pergamon Press, London, 1956, p. 85.
11. J. Shack, *J. Biol. Chem.*, 233 (1958) 677.
12. M. Pestemer and D. Brück, *Methoden der Organischen Chemie*, Band III/2, Georg Thiems Verlag, Stuttgart, 1955, p. 597.
13. A. M. Michelson, *Nature*, 182 (1958) 1502.
14. D. O. Jordon, E. Chargaff and J. N. Davidson, *The Nucleic Acids*, Vol. I, Academic Press, Inc. New York, 1955, p. 462.
15. J. W. Rowen, *Biochim. Biophys. Acta*, 10 (1953) 391.
16. M. Meselson, F. W. Stahl and J. Vinograd, *Proc. Natl. Acad. Sci., Wash.*, 43 (1957) 581.
17. B. Jacobson, *J. Am. Chem. Soc.*, 77 (1955) 2919.

STUDIES ON THE ROLE OF RIBONUCLEIC ACID IN THE GROWTH OF BACTERIA*

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SUMMARY

Measurements have been made of the protein, the RNA, and the DNA composition of cultures of *Aerobacter aerogenes* during balanced growth at different rates. The results indicate that at a given temperature the RNA/protein ratio of a culture is a direct function of the growth rate and that these two variables are linearly related at growth rates greater than 0.6 doublings per h. The RNA of these cultures is approximately 14 % soluble and 86 % ribosomal at all growth rates examined.

In the transition from a high to a lower growth rate the decrease in the rate of synthesis is immediate and marked for RNA and more gradual for protein and for DNA. The rate of RNA synthesis increases to the level characteristic of the new low growth rate only after the RNA/protein ratio has been reduced by preferential protein synthesis to the value normally observed during balanced growth at this new rate. In the transition from a low to a higher growth rate, the response of RNA synthesis is again more rapid than that of protein and DNA. The synthesis of RNA during this transition period proceeds at a rate greater than that finally to be established. The pattern of incorporation of radioactive guanine revealed that the usual proportion of soluble and of sedimentable RNA is made during this period, and the pattern of incorporation of radioactive leucine indicated that the sedimentable RNA consists of complete ribosomes.

The significance of these results in understanding the control of bacterial growth is discussed.

INTRODUCTION

Embarrassingly little is known of the role played by RNA in the growth and metabolism of bacteria. As in higher organisms, two classes of RNA are readily discernible: (1) "particulate RNA", comprising 80–90 % of the total RNA, and consisting of ribonucleoprotein particles (called ribosomes) of sedimentation velocity constants varying between 30 S and 100 S depending on the method of isolation, and (2)

The following abbreviations are used in this paper: RNA, ribonucleic acid; Tris, tris-(hydroxymethyl)aminomethane; DNA, desoxyribonucleic acid; TCA, trichloracetic acid; EDTA, ethylenediamine tetraacetic acid; DNAase, desoxyribonuclease.

* Preliminary reports of parts of this work were presented at the CIBA Foundation Symposium[1], London, 1958, and before the Society of American Bacteriologists, Saint Louis, May, 1959.

"soluble RNA", comprising 10–20% of the total RNA, and consisting of rather smaller molecules (mol. wt. of about 20,000) which are not protein-bound when isolated[2].

Soluble RNA appears to play the same role in bacteria as in higher organisms—that of an acceptor of activated amino acids in an early step of protein synthesis.* Furthermore, work with subcellular fractions of higher organisms[3] provides evidence that the particulate RNA of these systems plays a direct role in one of the final stages of protein synthesis. So far, however, the role of particulate RNA in microorganisms has remained a matter for conjecture.

Since the ribosomal RNA constitutes the bulk of the total RNA, one hint of the role of ribosomal RNA may be found in the observation that the rate of protein synthesis and the total amount of RNA are concomitant variables in most biological systems[4]. The present study was initiated to investigate this variation in a species of bacteria with the hope of elucidating the function of RNA. The procedures used were to measure the amounts of RNA, protein, and DNA in cultures growing at different rates, to determine the kinetics of the synthesis of these macromolecules during alterations in growth rate, and to describe the RNA in these studies in terms of its soluble and particulate components. The results shed light on the way the growth rate of a bacterial culture is controlled during balanced growth ("growth is balanced over a time interval if, during that interval, every extensive property of the growing system increases by the same factor"[5]), and during adjustment to changes in the external medium.

While this work was being completed the similar studies of SCHAECHTER, MAALØE AND KJELDGAARD[6,7] were published. Many of our observations are in close agreement with theirs.

MATERIALS AND METHODS

Organism

Two strains of *Aerobacter aerogenes* were isolated by the penicillin technique for use in these studies: strain 5-P14, which requires arginine and guanine for growth, and strain 32-P14, which requires leucine and guanine as growth factors. Both of these double auxotrophs were derived from the guanine-requiring strain P-14, which had been derived[8] from the wild strain, 1033.

Media

1. *Basal salts solution*. The inorganic salt composition (w/v) used in all the media was: $Na_2HPO_4 \cdot 7H_2O$, 1.34%; KH_2PO_4, 1.36%; $CaCl_2$, 0.0011%; $MgSO_4 \cdot 7H_2O$, 0.0246%; and $(NH_4)_2SO_4$, 0.2%. This solution (pH = 6.5) was prepared fresh at least twice weekly.

2. *Carbon sources*. The particular compound which was to serve as the major source of carbon and energy was prepared in either a 5% or a 10% solution and autoclaved separately. Sufficient amounts were then added to the basal salts solution to give a final concentration of 0.4%. Substances employed in this manner included glucose, *myo*-inositol, glycerol, and L-histidine.

3. *Growth factors*. The usual amounts of growth factors which were employed

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* Personal communication from F. GROS AND S. LACKS.

to provide an excess of each component were: guanine, 50 μg/ml (or, alternatively, an equivalent amount of guanosine); L-arginine, 100 μg/ml; and L-leucine, 50 μg/ml. The amino acids were kept in sterile solutions; guanine was kept in dilute KOH and autoclaved with the salt solution.

During short-term incorporation experiments the amount of the particular labelled growth factor was less than the above amounts, and is specified in each experiment.

4. *Supplements*. (a) Purine and pyrimidine ribosides. This supplement was a solution of adenosine, guanosine, uridine, cytidine, and thymidine, each at $6 \cdot 10^{-3}\,M$, and was used in the amount of 1 ml for every 20 ml of medium.

(b) Amino acid mixture. This solution consisted of a $10^{-2}\,M$ solution of the L-isomer of each of the following amino acids: arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, tyrosine, valine, alanine, glutamic acid, proline, aspartic acid, serine, and glycine. This mixture was used at a 1:100 dilution. Arginine and leucine were present in the supplement only when not already included in the medium as an essential growth factor.

(c) Vitamin mixture. This supplement was the commercial preparation BME Vitamin Mixture (Microbiological Associates, Inc.), and was used at a 1:100 dilution.

(d) Yeast extract-tryptone. This supplement was added to the medium to give a final concentration of 0.2 % yeast extract (Difco) and 0.2 % Bacto-Tryptone (Difco).

(e) Casamino acids. This commercial preparation (Difco) of hydrolyzed casein was used to give a final concentration of 0.2 %.

Method of cultivation and measurement of growth

For all experiments cells were grown at 35° on a platform shaker with an excursion of 5 cm and a frequency of 90 excursions/min. Cultures were contained either in Erlenmeyer flasks filled to no more that one-tenth their capacity, or in special wide-bottom flasks that could contain up to 500 ml. Using this procedure, and employing cultures that contained no more than 350 μg dry weight of cells per ml, conditions of aerobiosis could easily be maintained.

A few experiments employed a chemostat kindly lent to us by Dr. LUIGI GORINI.

Growth was usually followed by measuring the turbidity of the culture in a Klett-Summerson photoelectric colorimeter (Filter no. 42) which had been calibrated and standardized with exponential-phase cells of this organism dried to constant weight. The measurements were expressed either as dry weight of cells per ml of culture, or simply as Klett units. This method probably introduces an error due to cell-size differences under different conditions, but none of our results are dependent for their validity on exact knowledge of the dry weight of a culture.

Growth rates were expressed as k, the number of doublings per hour.

Fractionation of cells into soluble and particulate components

Cultures were chilled rapidly by pouring them into pre-chilled flasks immersed in an ice-salt bath. They were then centrifuged, usually in plastic bottles, in a refrigerated International centrifuge. The culture fluid was then decanted, the walls of the containers rinsed, and the cells suspended (with or without additional washing) in 11 ml of a buffer (pH 7.4) composed of 0.001 M Tris and 0.01 M $MgCl_2$; DNAase (Pabst) was added to a concentration of approximately 10 μg/ml. This suspension was then treated in a Raytheon 10 kc magnetostrictive oscillator for 4 min. Alternatively, the cells were ground with about 3 parts of alumina powder in the cold and extracted with 10 ml of the same buffer. In either case, the resulting extract was centrifuged at 10,000 rev./min in a Servall Superspeed centrifuge for 20 min, and the clear supernatant fluid carefully decanted. This preparation is referred to as an unfractionated, cell-free extract.

Such an extract was then centrifuged in a Spinco Ultracentrifuge (preparative model) at 38,000 rev./min for 90 min. This procedure yielded a crystal-clear supernatant liquid and a compact, gummy pellet. The liquid was carefully decanted and the pellet suspended in the Tris–$MgCl_2$ buffer. For the purpose of these investigations, no further purification steps were deemed necessary.

This procedure is based on that developed by TISSIÈRES AND WATSON[2].

Colorimetric analyses

The colorimetric assays employed were: the orcinol method for RNA[9], the phenol method for protein[10], and the indole method for DNA (CERIOTTO[11] as modified by KECK[12]). To assay these components of whole cultures, 5–10 ml of culture were sampled and brought to 5 % TCA. The samples were stored overnight at 4°, then centrifuged and the pellet washed once with cold 5 % TCA. The washed pellet was resuspended in exactly 5 ml of 5 % TCA, heated at 100° for 30 min, the mixture cooled, centrifuged, and the supernatant fluid carefully decanted. Aliquots of the supernatant fluid were assayed directly for RNA and DNA. The pellet was dissolved in 1 N NaOH, diluted 10 fold, and assayed for protein.

The procedure was similar when these assays were performed on cell-free extracts. The nucleic acids and proteins were precipitated and washed with cold 5 % TCA, and extracted with hot 5 % TCA; the hot TCA extract was assayed for ribose and the residual precipitate was dissolved in 1 N NaOH and assayed for protein.

Despite the many pitfalls of these colorimetric methods, we have found reasonably good agreement between them and independent methods of analysis (260 mμ absorption for nucleic acid, guanine incorporation for nucleic acid, and amino acid incorporation for protein).

Measurement of [8-^{14}C]*guanine incorporation*

(a) *Differential incorporation into RNA and DNA*. The culture was chilled and the cells (~ 75 mg) were harvested and washed free of culture fluid by successive centrifugation. The cells were then extracted with cold 5 % TCA, twice with ethanol–ether (3:1), and finally dissolved in 1.0 ml of 1 N KOH. After 1 h of hydrolysis at 37° the mixture was chilled, neutralized with 1.5 N HCl, and acidified to 5 % TCA. The precipitate (consisting of protein and DNA) was washed once with cold 5 % TCA and hydrolyzed in 5 % TCA at 100° for 30 min. The mixture was centrifuged and the supernatant fluid concentrated and fractionated on a descending paper chromatogram in isopropanol–HCl–H_2O[13]. The guanine spot was eluted with 0.1 N HCl, the concentration determined by u.v. absorption in a Beckman DU spectrophotometer, and an aliquot counted in a gas-flow Geiger counter.

The supernatant liquid containing hydrolyzed RNA was made 0.1 N with respect to HCl and twice extracted with 5.0 ml of ether to remove the TCA. The pH was adjusted to 4 and 100 mg of Darco charcoal added. The suspension was shaken for 30 min, then centrifuged and the charcoal washed once with 2.0 ml of H_2O. Next, 3 ml of 0.01 M EDTA (brought to pH 7.5 with NH_4OH) was added and the mixture was shaken and then centrifuged. The charcoal was then treated with ethanol–H_2O (50 %) containing 1 % NH_3 for 20 min with shaking. The eluate, which contained the nucleotides, was then subjected to paper electrophoresis in 0.04 M citrate at pH 3.4–3.5 for 2 h with a voltage gradient of 33 V/cm. The guanylic acid spot was eluted, the concentration measured by u.v. absorption in the Beckman DU spectrophotometer and an aliquot counted in a gas-flow Geiger counter.

(*b*) *Incorporation into soluble and particulate RNA*. Following separation of the cell-free extracts into soluble and particulate fractions (as described above), the two fractions were brought to 5 % TCA and centrifuged. The supernatant fluids were discarded and the pellets washed once with cold 5 % TCA. Each pellet was then hydrolyzed for 1 h at 37° in 1 ml of 1 N KOH. The hydrolysates were carefully neutralized with perchloric acid, the precipitate $KClO_4$ removed, and then sufficient perchloric acid added to give an additional concentration of 5 %. The precipitate (consisting of protein, and any DNA that has escaped the action of the DNAase in the Tris buffer) was discarded. The supernatant fluid was neutralized with KOH, the precipitated $KClO_4$ removed, and the supernatant concentrated and fractionated by paper chromatography using the isopropanol–HCl–H_2O solvent described previously. The guanylic acid spots were eluted, the concentration of the eluates was measured spectrophotometrically, and their radioactivity was determined by the methods described above.

Measurement of L-[^{14}C]*leucine incorporation into proteins*

When incorporation of L-leucine into soluble and particulate protein was to be measured, cell-free extracts of the cultures were prepared and fractionated by the procedures described above. Each fraction was then acidified with 5 % TCA containing 2 mg of unlabelled L-leucine/ml and heated at 90° for 20 min. The residual precipitates were washed once with the same reagent and then dissolved in 1 ml of 1 N NaOH containing 2 mg of L-leucine/ml. This mixture was acidified by the addition of 1 ml of 20 % TCA and the resulting precipitate extracted twice (with warming) by 5-ml portions of each of the following: acetone, alcohol–ether (3:1), and finally, ether. The air-dried proteins were then dissolved in 1 N NH_4OH and aliquots taken for estimation of protein by dry weight and by colorimetric assay, and for measurement of radioactivity. The amount of protein plated on planchets for counting never exceeded 100 μg/cm^2, and corrections for self-absorption were unnecessary.

This method is a modification of that of MANDELSTAM[14].

Chemicals

L-[^{14}C]leucine (uniformly labelled), 5.7 μC/μmole, was obtained from the Nuclear Chicago Corporation (Chicago). [8-^{14}C]Guanine, 1.23 μC/μmole was obtained from Isotope Specialities Company ,Inc. (Burbank, California).

All purine and pyrimidine bases and ribosides, and all amino acids were obtained from the California Corporation for Biochemical Research (Los Angeles) and were of a purity equivalent to their A Grade.

All other organic and inorganic chemicals were of reagent grade.

RESULTS

Variation of the RNA content of A. aerogenes

Many microorganisms exhibit a marked fluctuation in their RNA content depending on the conditions of cultivation. To determine the behavior of *A. aerogenes*, strains 5-P14 and 32-P14, measurements were made of the RNA, DNA, and protein of cultures of these strains grown in different ways.

Initially, a stationary phase culture was defined simply as one which had been incubated aerobically for 18 h in the minimal medium described above, with glucose as the major source of carbon and energy. The first line of Table I presents the composition of such a culture assayed by the methods already described. These values should be compared to those in the second line which were obtained when the measurements were made on cells taken from the exponential phase of growth in a medium of the same composition. The most striking difference between the two cultures is in their content of RNA. Whether the RNA level is expressed in relation to mass of protoplasm (measured turbidimetrically), protein, or DNA, the RNA of exponential phase cultures is three to four times that of stationary phase cultures.

TABLE I

THE COMPOSITION OF *Aerobacter aerogenes*, STRAIN 5-P14, DURING THE STATIONARY PHASE AND DURING EXPONENTIAL GROWTH ON DIFFERENT SOURCES OF CARBON AND ENERGY

Carbon source	*Growth rate constant (k)*	*Composition of culture of standard turbidity* (μg/ml)*		
		Protein	*DNA*	*RNA*
Glucose	(stationary)	55	6.0	8.5
Glucose	0.97	51	4.5	27.5
Glycerol	0.72	53	4.2	21.0
Inositol	0.50	60	5.0	18.0
Histidine	0.29	47	4.8	14.0

These results were obtained using the colorimetric techniques described under MATERIALS AND METHODS. See text for details of the experimental procedure. Each figure represents the average of three or more determinations.

* A Klett reading of 150.

Measurements were next made to ascertain whether exponential phase cultures possess the same high RNA content regardless of the method of cultivation. Accordingly, samples were taken from cultures in the exponential phase of growth with glycerol, *myo*-inositol, or L-histidine serving as the major source of carbon and energy and were analyzed for RNA, DNA, and protein. The results of these analyses are presented in Table I also. It may be seen that, again, there is little variation in the ratio of DNA to protein among these cultures. The RNA content, however, shows considerable variation under the different conditions, though it is always significantly above the level of the stationary-phase culture.

The results summarized in Table I provoke two questions: (1) how is the RNA content of growing cultures of bacteria reduced when they enter the maximum stationary phase, and (2) how is it related to the chemical composition of the medium? Experiments relevant to these questions are described in the next two sections.

Change in RNA levels of cultures entering the stationary phase

In answering the first question, involving cells which are not growing exponentially, it seemed desirable to specify the condition restricting growth. For this reason it was decided to investigate the effect of different nutritional limitations on the composition of strain 5-P14. Experiments were performed in which exhaustion from the medium of one of the following components resulted in a transition from exponential growth to a stationary condition: (1) the essential amino acid, L-arginine, (2) the essential purine, guanine, and (3) the major source of carbon and energy, glucose.

All three of the experiments were performed in the same way. Exponential phase cells were inoculated (to a final concentration of about 40 μg dry weight per ml) into media containing the usual excess amounts of all components but one. The particular limiting factor was added in an amount calculated to support growth only to about 200 μg dry weight of cells per ml. Growth was followed turbidimetrically, and samples were taken at various times to be analyzed for RNA, DNA, and protein by the usual methods. In Fig. 1 the results are plotted from the time when the first deceleration of growth (as measured turbidimetrically) was noted. The values are expressed in terms of the levels of all components being 1.00 at this (somewhat arbitrary) time.

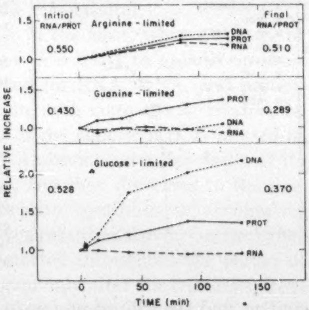

Fig. 1. The relative increases of DNA, RNA, and protein in cultures of *A. aerogenes*, strain 5-P14, entering the maximum stationary phase because of limiting amounts of L-arginine, guanine, or glucose. The excess and the limiting concentrations, respectively, of these medium constituents were: L-arginine, 100 μg/ml and 10 μg/ml; guanine, 50 μg/ml and 5 μg/ml; glucose, 2000 μg/ml and 250 μg/ml. RNA, DNA, and protein were measured by colorimetric methods, and the increases in these components, relative to their values when growth ceased to be exponential, are plotted against time. The numbers to the left of the curves are the RNA/protein ratios at the time when growth ceased to be exponential; the numbers to the right are the RNA/protein ratios of the final samples.

It is apparent that the kinetics characteristic of decelerating growth depend partially on the nature of the limiting factor. (1) When arginine became limiting there was a very rapid and simultaneous deceleration in the rate of net synthesis of all three of these major cell components; the RNA/protein ratio 125 min after the arginine had become limiting had changed very little (0.55 to 0.51). (2) In the culture limited by the guanine concentration, RNA and DNA ceased increasing at the time that growth (measured turbidimetrically) slowed down, but the synthesis of protein continued at a slow rate throughout the 125-min period studied, dropping the RNA/protein ratio from 0.43 to 0.29. (3) In the culture in which glucose was limiting, there was an immediate cessation of net RNA synthesis a full 40 min before the synthesis of DNA was affected. The protein of this culture continued to increase throughout the experiment at a slow but easily measured rate, reducing the RNA/protein ratio from 0.53 to 0.37.

These results indicate that the low RNA/protein ratios of stationary phase cultures can be due, at least in part, to a preferential increase in the protein of the culture after RNA increase has ceased.

RNA content of exponential-phase cells

Experiments were next undertaken to determine the effect of the chemical composition of the medium on the RNA level of exponential-phase cultures.

Cultures of strain 5-P14 were grown under each of ten different conditions for a minimum of three generations, and then samples were taken and analyzed for RNA, DNA, and protein. The methods of cultivation differed from each other in one or more of the following ways: (a) the nature of the major source of carbon and energy, (b) the presence or absence of an exogenous supply of amino acids, and (c) the rate of supply of the growth-essential amino acid, L-arginine. The latter parameter was varied by means of a chemostat.

Each method of cultivation resulted in a different growth rate and in a different RNA/protein ratio of the culture. As shown by Fig. 2, the data indicate a direct relation between the RNA content of a culture and its growth rate. Since all of the cultures were in balanced growth, this relation is also true between the RNA content and the rate of protein synthesis. Starting at the highest growth rate (k = 1.10) and proceeding to slower rates, there is a proportional decrease in RNA content for every decrease in growth rate until at a growth rate of $k = 0.6$ and below, a minimum level of RNA is approached. The smooth curve described by the data indicates that the composition of the medium determines the level of RNA only insofar as it determines the growth rate of the cells.

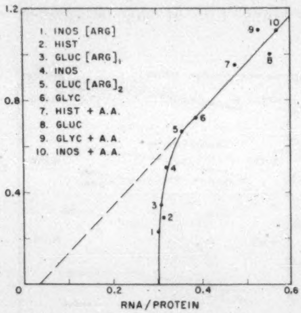

Fig. 2. The relation between growth rate and RNA content of *A. aerogenes*, strain 5-P14. The growth rate is expressed as the value of the growth rate constant, k (doublings/h), and the RNA content is expressed as the RNA/protein ratio based on colorimetric measurements. Each of the numbered points in the figure represents the average of at least two experiments, analyzed in duplicate. The cells were grown in the various media for at least three generations before any samples were taken. Except where indicated otherwise, all of the media contained basal salts supplemented with the usual excess amounts of guanine and L-arginine. The major carbon and energy sources, supplied at 0.2% final concentration, were: (1) *myo*-inositol, with L-arginine supply limited by the chemostat; (2) L-histidine; (3) glucose, with L-arginine supply limited by the chemostat; (4) *myo*-inositol; (5) glucose, with the supply of L-arginine by the chemostat greater than in (3) but still limiting; (6) glycerol; (7) L-histidine plus hydrolyzed casein; (8) glucose; (9) glycerol plus hydrolyzed casein; (10) *myo*-inositol plus hydrolyzed casein.

Having established the steady-state relationship between growth rate and RNA content, it became important to know the manner in which an exponentially growing culture adjusts its RNA content when undergoing an increase or a decrease in its growth rate. Studies were first made of the kinetics of RNA synthesis during a decrease in growth rate caused by a change in the major source of carbon and energy.

RNA synthesis during diauxic growth

Cells of this organism grow very well when *myo*-inositol is the sole source of carbon and energy, metabolizing this substance by a series of inducible enzymes[15]. The synthesis of these enzymes is prevented during growth on glucose, even when the inducer, *myo*-inositol, is present[16]. This repression of enzyme formation gives

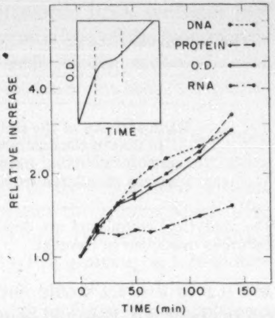

Fig. 3. The relative increases of DNA, RNA, protein, and O.D. in a culture of *A. aerogenes*, strain 5-P14, during a diauxic lag. The cells were grown in 250 ml of the basal salts solution supplemented with guanine (50 μg/ml) and L-arginine (100 μg/ml), and containing glucose (600 μg/ml) and *myo*-inositol (2000 μg/ml) as major sources of carbon and energy. The inset to the figure describes the diauxic growth curve of this culture, as measured by its O.D. During the period included by the dashed lines in the inset, when the culture was adjusting from growth on glucose to growth on *myo*-inositol, samples were taken and assayed for RNA, DNA, and protein by the methods previously described. The results are plotted in the main part of the figure as relative increases in these components as a function of time. The increases are relative to the individual values at 0 time, shortly prior to the beginning of the diauxic lag.

rise to the familiar diauxic type of growth curve when the culture is supplied with both glucose (in limiting amounts) and *myo*-inositol. As the inset of Fig. 3 shows, there is a period of exponential growth (during which it can be demonstrated that the cells are growing at the expense of the glucose exclusively) followed by a lag period which lasts while the cells synthesize the appropriate inducible enzymes. Finally there is a phase of exponential growth on the *myo*-inositol.

During the period included by the dashed lines in the inset, when a culture of strain 5-P14 was adjusting from growth on glucose ($k = 0.93$) to growth on *myo*-inositol ($k = 0.50$), samples were taken and analyzed for RNA, DNA, and protein by the usual colorimetric methods. The increase in each component, relative to the value at 0 time (shortly prior to the beginning of the diauxic lag), is plotted in the main part of the figure. It can be seen that DNA accumulation continues at its original rate during the first part of the lag period, and then decreases to the new rate characteristic of growth on *myo*-inositol. Protein continues to accumulate throughout the lag period, though at a slightly lower rate than DNA. On the other hand, very little net increase in RNA could be detected throughout the period of diauxic lag; RNA began to increase more rapidly again only after the transition from glucose metabolism to *myo*-inositol metabolism had been accomplished.

The failure to observe any net increase in RNA during the diauxic lag could be the result of two processes: (1) a cessation of RNA synthesis, or (2) a breakdown of RNA masking a continued synthesis. To distinguish between these alternatives the experiment was repeated, and a small amount of [8-^{14}C]guanine (5.2 μmoles) of specific activity (1.23 μC/μmole) was added to the guanine-containing culture (20 μg/ml) at the beginning of the lag phase; the cells were harvested at the end of the lag phase (1 h) and their nucleic acids were fractionated by the methods described above. The molar radioactivity of the RNA-guanine and of the DNA-guanine was determined and compared with the molar radioactivity of the guanine present in the growth medium. The results (Table II) indicate that much less guanine was incorporated into RNA than into DNA. Therefore, despite an incorporation corresponding to a synthesis of 11 %, the marked decrease in accumulation of RNA during the diauxic lag seems to be caused mainly by a decreased rate of synthesis.

Preferential RNA synthesis during increases in growth rate

It can be seen by reference to Fig. 2 that an increase in the rate of growth from

TABLE II

THE INCORPORATION OF [8-^{14}C]GUANINE INTO THE NUCLEIC ACIDS OF *Aerobacter aerogenes*, STRAIN 5-P14, DURING A DIAUXIC LAG

	RNA guanine	*DNA guanine*
Radioactivity of the isolated guanine relative to that in the medium (97,000 counts/min/µmole)	0.103	0.330
Increase calculated from the radioactivity	11 %	49 %
Increase calculated from colorimetric measurements	7 %	40 %

The analytical techniques are described under MATERIALS AND METHODS. The experimental procedure is described in the text.

$k = 0.2$ to $k = 1.2$ would entail an increase in the RNA/protein ratio from about 0.3 to about 0.6. It would be of interest to know whether the increased RNA/protein ratio is a prerequisite for the increase in growth rate or whether it is simply a consequence of the increased growth rate. One approach would be to determine whether one detectably preceded the other. Fortunately, it proved relatively simple to demonstrate a temporal difference between an increase in the level of RNA in a culture and an increase in the rate of protein synthesis.

The appropriate experiment was first performed by the addition of a mixture of yeast extract and tryptone (see MATERIALS AND METHODS for the composition) to a culture of strain 5-P14 growing on L-histidine as the major source of carbon and energy. Aliquots were taken at various times before and after the supplementation and analyzed for RNA, DNA, and protein by the usual colorimetric methods.

The results are shown in Fig. 4, where relative increases in RNA, protein, and DNA (with all values assumed to be 1.00 at the time of addition of the supplements) are plotted on a logarithmic scale against time. The samples taken at 0 and at 50 min show that during the growth on L-histidine with a $k = 0.28$ there was balanced synthesis of RNA, DNA, and protein. At 50 min the supplement was added, and almost immediately there was a great increase of synthesis of RNA. This rapid synthesis (at a rate temporarily greater than the rate which was to be established) continued for about 25 min, during which time the rate of synthesis of DNA and of

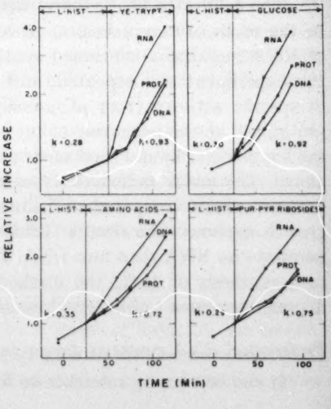

Fig. 4. The relative increases of DNA, RNA, and protein in cultures of *A. aerogenes*, strain 5-P14, during accelerating growth following the addition of (a) yeast extract-tryptone, (b) glucose, (c) a mixture of amino acids, or (d) a mixture of purine and pyrimidine ribosides to an L-histidine medium. See the text for details of the experimental procedure, and MATERIALS AND METHODS for the composition of the media and the supplements.

protein increased only slightly. By this time the preferential RNA synthesis had raised the RNA content of the culture to the level characteristic of the new growth rate, and the rate of synthesis of DNA and of protein then quickly reached the new value, and the synthesis of all three macromolecules proceeded at the same differential rate ($k = 0.93$).

The results of this experiment make it unlikely that high RNA content is simply a consequence of fast growth rather than a necessary antecedent. But if this conclusion is correct, then one should be able to vary the nature of the supplement without changing the results of the experiment. Fig. 4 also shows the results which were obtained when (a) glucose, (b) a mixture of amino acids, and (c) a mixture of purine and pyrimidine ribosides were added to cells of strain 5-P14 growing on L-histidine. In all cases the attainment of the new high growth rate was preceded by a rapid, preferential synthesis of RNA. It was inconsequential whether the supplement consisted mainly of precursors of nucleic acid or of protein.

It now seemed of interest to investigate the nature of the RNA which is synthesized under these conditions.

Particulate and soluble components of A. aerogenes

The bulk of the RNA in bacterial cells exists in the form of ribonucleoprotein particles (ribosomes) of high molecular weight, and with sedimentation coefficients ranging from 30 S to 100 S, depending on the particular conditions employed to prepare them. The remainder of the RNA consists of low molecular weight units which are not sedimented with the ribosomes and which are soluble down to a pH of 5. This fraction has been termed the "soluble RNA" and has been implicated in the incorporation of amino acids into proteins.

Using the procedure of TISSIÈRES AND WATSON (see MATERIALS AND METHODS) to fractionate the RNA of cultures of our strains of *A. aerogenes*, it was determined that about 15 % of the total RNA is not sedimented by a force of 100,000 × g acting for 120 min, while the remainder forms a compact and easily isolated pellet.

Such a fractionation was performed on two separate cultures of strain 5-P14: one growing on L-histidine and one on glucose. Aliquots (5 ml) were taken periodically from each culture for RNA and protein determinations and the two exponential-phase cultures (each of 250-ml volume and with a bacterial density of 250 μg dry weight per ml) were chilled rapidly, harvested by centrifugation, and washed once with the Tris–Mg buffer. Each pellet was ground with alumina and the resulting extracts were fractionated into soluble and particulate components. Each fraction was then assayed colorimetrically for RNA and for protein. (See MATERIALS AND METHODS.)

The results of these analyses were combined with the values for the total RNA and total protein of the respective cultures, and the soluble and particulate constituents of the original cultures were calculated. The results are given in Table III and may be summarized as follows:

(1) Rapidly growing cells have both more soluble and more particulate RNA; the ratio of soluble to particulate RNA seems to be independent of the growth rate.

(2) The ratio of the protein to the RNA of the particulate fraction seems to differ significantly between cultures growing at different rates. The range of values, however, agrees very well with that found by other investigators[2].

TABLE III

PARTICULATE AND SOLUBLE COMPONENTS OF *Aerobacter aerogenes*, STRAIN 5-P14, GROWING AT DIFFERENT RATES

Cell constituent	Fraction*	Major carbon source in growth medium	
		Glucose ($k = 0.90$)	L-histidine ($k = 0.31$)
RNA	soluble, μg/ml	3.6	2.2
	particulate, μg/ml	22.8	12.8
	soluble/particulate	0.16	0.17
Protein	soluble, μg/ml	43.3	43.2
	particulate, μg/ml	11.7	7.8
	soluble/particulate	3.7	5.5
Ribosomes	total, μg/ml**	34.5	20.6
	protein/RNA***	0.55	0.66

* All values are expressed as μg/ml of a culture of standard turbidity (Klett reading of 150). See text for experimental details.

** Calculated as the sum of the particulate protein and the particulate RNA.

*** Calculated as the ratio of the particulate protein to the particulate RNA.

With this information in hand, it was now possible to return to the original problem: the nature of the RNA made preferentially during accelerating growth.

Nature of the RNA made during accelerating growth

The incorporation of [8-^{14}C]guanine into the RNA of a culture of strain 5-P14 during accelerating growth was compared with the incorporation into a control culture. Acceleration was achieved by the addition of a rich supplement (amino acids, purine and pyrimidine ribosides minus guanosine, vitamins, and glucose) to a culture growing relatively slowly on *myo*-inositol (with arginine (100 μg/ml) and guanine (20 μg/ml) present as required growth factors). At the time of supplementation, a small amount of [8-^{14}C]guanine (4.56 μmoles) of high specific activity (1.23 μC/μmole) was added to the culture, and an identical amount added to a control culture which received no supplement.

After 20 min of continued incubation the two cultures were chilled and harvested by centrifugation. Cell-free extracts were prepared and fractionated in the usual manner. Colorimetric analyses for RNA were performed on the soluble and the particulate fractions of the two extracts, as well as on samples of the two cultures taken at 0 time and at 20 min. The results of these measurements are shown in Part A of Table IV, and in Part C these results have been used to calculate the increase in soluble and in particulate RNA in the two cultures.

The specific activity of the guanine in the soluble and in the particulate fractions was measured by the usual methods, with the results that are presented in Part B of Table IV. Calculation of the increase in the two fractions of both cultures are given in Part C.

The colorimetric and the isotope incorporation data are in excellent agreement. They show a 20–25 % increase in both the soluble and the particulate RNA in the control culture, and a 40–45 % increase in both fractions of RNA in the experimental culture.

TABLE IV

THE NATURE OF THE RNA SYNTHESIZED BY *Aerobacter aerogenes*, STRAIN 5-P14, DURING ACCELERATING GROWTH

A. Colorimetric assay of the total RNA and of the ratio of soluble to particulate RNA during accelerating growth.

Time min	*Ribonucleic acid of control culture*		*Ribonucleic acid of experimental culture*	
	total RNA µg/ml	*sol./part. RNA*	*total RNA (µg/ml)*	*sol./part. RNA*
0	42.8	0.15*	42.8	0.15*
20	52.0	0.17	60.5	0.15

* Separate experiment.

B. Incorporation of [8-^{14}C]guanine into the soluble and the particulate fractions of RNA during accelerating growth.

Flask	*Specific activity (counts/min/µmole)*		
	guanine in medium	*guanine in soluble RNA*	*guanine in particulate RNA*
Control	53,000	10,800	9,300
Experimental	53,000	16,100	15,600

C. Calculated syntheses of soluble and of particulate RNA.

Culture	*Component*	*Increase calculated from colorimetric measurements (data of Part A.)*	*Increase calculated from radioactivity (data of Part B.)*
Control	soluble RNA	21 %	25 %
	particulate RNA	21 %	23 %
Experimental	soluble RNA	41 %	43 %
	particulate RNA	41 %	42 %

Protein synthesis during accelerating growth

Since the RNA made during accelerating growth proved to be, like that made during balanced growth, mostly (85 %) particle-bound, it became of interest to know whether the protein moiety of the particles is also synthesized preferentially during accelerating growth.

Two 300-ml exponential-phase cultures of strain 32-P14 were prepared with L-histidine as the major carbon and energy source and with the required growth factors, guanine (50 µg/ml) and L-leucine (10 µg/ml). At 0 time a small amount (1.2 µmoles) of uniformly-labelled L-[^{14}C]leucine of high specific activity (5.7 µC/µmole) was added to each of the two cultures. One of the cultures received a simultaneous supplement of amino acids, purine and pyrimidine ribosides, vitamins, and glucose, while the other received no supplement. Small samples of both cultures were taken for colorimetric analyses at 0 time and 25 min later, when both cultures were harvested and cell-free extracts prepared and fractionated as before. This time, however, the protein of the two fractions was isolated (see MATERIALS AND METHODS) and the specific activities of the whole proteins were determined as counts per minute per milligram of dried protein. From these activities it was possible to calculate the specific activities of the L-leucine in the proteins (Table V) for the following reasons: (1) strain 32-P14 must use the L-leucine presented to it for protein synthesis as it is incapable of manufacturing any, (2) L-leucine added to the medium appears in protein exclusively as leucine, and (3) the average leucine content of the soluble and particle-bound proteins of this organism has been measured and found to be 8.5 % (w/w) at two different growth rates[14].

TABLE V

THE SYNTHESIS OF SOLUBLE AND OF PARTICULATE PROTEIN BY *Aerobacter aerogenes*, STRAIN 32-P14, DURING ACCELERATING GROWTH

Culture	*Time*	*Colorimetric assay*				*Incorporation of [^{14}C]leucine*			
		Total RNA		*Total protein*		*Specific activity of bacterial protein* (counts/min/µg leucine)*		*Increase in protein based on specific activity*	
		µg/ml	*increase*	*µg/ml*	*increase*	*Soluble*	*Particulate*	*Soluble*	*Particulate*
Control	0 min	22.6		86		0	0		
	25 min	25.9	14.6 %	99	15.1 %	197	212	21 %	23 %
Experimental	0 min	24.8		86		0	0		
	25 min	46.9	89 %	111	29 %	201	432	22 %	62 %

* The specific activity of the [^{14}C]leucine in the medium of both cultures was 1130 counts/min/µg.

Colorimetric analyses of the aliquots taken at the time of supplementation and at the time of harvesting showed that the control (unsupplemented) culture increased about 15 % in total RNA and in total protein. Leucine incorporation indicated a somewhat higher rate of synthesis (about 25 %) for both the soluble and particulate protein fractions. (This discrepancy is assumed to be an analytical artifact.) The experimental flask exhibited a dramatic 89 % increase in total RNA and a 29 % increase in total protein, measured colorimetrically. Comparing the specific activities of the soluble and particulate protein fractions in this culture, it can be seen that there has been a preferential synthesis of particle-bound protein; while the soluble fraction increased no more than did that of the control culture (22 %), the particle-bound fraction increased almost three times as much (62 %) as did that of the control. Therefore, we may conclude that the rapid synthesis of particulate RNA during accelerating growth is accompanied by a preferential synthesis of particulate protein. This experiment has been repeated several times, and has been performed using different carbon and energy sources and different labelled amino acids, always with similar results.

This demonstration that the protein moiety of the ribosomes is made concomitantly with the RNA during accelerating growth (Table V) shows, in fact, that an 89 % increase in RNA was accompanied by only a 62 % increase in ribosomal protein. A partial explanation of this difference stems from the fact that the protein/RNA ratio of the ribosomal fraction prepared from rapidly growing cells is consistently 10–20 % lower than that of the same fraction of slowly growing cells (*cf.* Table IV). This difference would almost account for the discrepancy (25 %) observed in the incorporation studies. Since no attempt was made to purify the ribosomal preparations beyond that described in MATERIALS AND METHODS, it is possible that the variation of the protein/RNA ratio of these preparations is due to small amounts of contaminating non-ribosomal protein. Other explanations are conceivable, but further information would be required to discuss them profitably.

DISCUSSION

It is by now a well established fact that growing bacterial cultures are richer in RNA than are non-growing cultures. WADE AND MORGAN[18], for example, found that the level of RNA in "dividing cells" was from 1.3 to 16.5 times higher than in "resting cells" of the dozen bacterial species they examined. Our strain of *A. aerogenes* exhibits a three-fold variation in its RNA level depending on the physiological condition of the culture. Such variation in a major constituent of the cell is of particular interest since the physiological role of this component is not fully understood.

In view of the reported stability of RNA during growth[19, 20], it seemed of interest to learn how the RNA content is reduced when cultures enter the stationary phase. It was realized, however, that the stationary phase does not represent a unique physiological state. Cultures may have ceased increasing for any one (or more) of a number of reasons: exhaustion of an organic or an inorganic growth factor, exhaustion of the major carbon and energy source(s), limitation of oxygen, or accumulation of toxic metabolic products. We have studied three conditions restricting growth: exhaustion of a required amino acid, of a required purine, and of the major carbon and energy source. When growth became limited by the exhaustion either of the purine or of the major carbon and energy source, the net synthesis of RNA was reduced much more drastically than was the net synthesis of protein or of DNA. Thus, the RNA/protein ratio became gradually reduced during the transition from exponential growth to a stationary phase by means of a continued synthesis of protein after RNA accumulation had ceased. When an amino acid became limiting, however, there was an abrupt and virtually simultaneous reduction of the synthesis of RNA, protein, and DNA.

The findings with carbon source or purine limitation are similar to those of SCHMIDT *et al.*[21] who observed that phosphate exhaustion in a yeast culture results in a continued, slow synthesis of protein after RNA synthesis has ceased. These results, together with our own, make it clear that cultures can undergo a considerable reduction in RNA content simply by a continued synthesis of other cellular components (*e.g.*, protein and DNA) after RNA accumulation has halted.

The RNA/protein ratio of stationary phase cells predicted by Fig. 2 is about 0.30, while that observed in an overnight culture (Table I) is about 0.16. This discrepancy is probably due to the fact that when cells are incubated for long periods of time (10 h or more) under non-growing conditions there is an actual destruction of some RNA[21–23]. In general, however, one observes little or no net destruction of RNA for several hours after a culture has ceased exponential growth due to the exhaustion of some constituent of the medium.

The next series of experiments showed that there is not a unique RNA level which is characteristic of a "growing" cell as compared to that of a "resting" cell, but rather that the RNA content of growing cultures can vary over a wide spectrum of values. This variation is shown by our work, as well as that of many others[6, 7, 18, 24–27] to be associated with variations in the growth rate of the cultures. Different authors have described the relationship between RNA content and growth rate in microorganisms in quantitatively different terms, but in general the following conclusion can be (and has been) drawn: at a given temperature the RNA content of a culture is a direct function of the growth rate and is affected by the composition of the medium only in so far as the latter influences the growth rate. From our information about *A. aerogenes* we may draw the additional conclusions: (1) in rapidly growing cultures ($k > 0.60$) the relation between growth rate and RNA/protein ratio is approximately linear, and (2) the amount of soluble RNA (that which is not sedimented in 120 min by $100{,}000 \times g$) seems to be a constant proportion (about 14 %) of the total amount of RNA in a culture, regardless of the growth rate.

These measurements of the RNA, DNA, and protein content of cultures in balanced growth at different rates were followed by studies of the transition of cultures from one rate of balanced growth to another. When cells are caused (by changing their carbon source) to slow down their rate of growth, the transition is characterized by a marked decrease or complete cessation of RNA accumulation, followed by a more gradual slowing down of protein and of DNA synthesis (Fig. 3). RNA formation resumes only after the RNA/protein ratio has been reduced to that characteristic of the new low growth rate. The synthesis of RNA appears, therefore, to be much more sensitive to the decreased supply of energy and of carbon compounds than is that of the other two macromolecules studied.

When, by the addition of various enrichments to the medium, a culture is caused to accelerate its growth rate, the transition is characterized by a rapid acceleration of RNA synthesis to a rate temporarily greater than that to be finally attained in the new environment (Fig. 4). The pattern of incorporation of radioactive guanine during this period revealed that the usual proportion of soluble and of sedimentable RNA was being made. The pattern of incorporation of radioactive leucine indicated that the sedimentable RNA synthesized during this period consisted of complete ribosomes. After this rapid, preferential synthesis of RNA has raised the level of RNA in the cells to that characteristic of the new high growth rate, the synthesis of protein and that of DNA are accelerated to the new rate and balanced growth ensues. Thus, quite analogous to the results obtained during a period of decreasing growth rate, the synthesis of RNA is the reaction most immediately sensitive to a sudden enrichment of the medium. This conclusion is strengthened by the finding that the exact biochemical nature of the enrichment used to accelerate growth is of no importance in determining the response of the cells; the same rapid increase in RNA formation preferential to that of protein occurs whether the supplement consists of amino acids or of purine and pyrimidine ribosides.

Two implications of our findings are of general significance and deserve comment. The first concerns the role of RNA in the biosynthesis of protein, and the second the metabolic control of the biosynthesis of RNA.

It has been postulated that the ribosomal RNA plays the role of a passive template in protein synthesis[28]. According to this concept, the rate of protein synthesis would be determined by the RNA content of the cell and by the concentration of the activated amino acids which are to be polymerized. Were these latter present in sufficient concentration to saturate the template, the rate of protein synthesis would be directly proportional to the RNA content of the cellular mass. This theoretical picture accurately describes the situation in cultures of *A. aerogenes* growing at rates greater than $k = 0.6$, where the rate of protein synthesis is directly proportional to the RNA content.

On the other hand, if the concentration of the activated amino acids were to be below that needed to saturate the template, then an increase in the supply of these precursors would result in an increased rate of protein synthesis without a concomitant increase in the RNA content of the cellular mass. This picture accurately describes the situation in cultures of *A. aerogenes* growing at rates less than $k = 0.6$, where the rate of protein synthesis is independent of the RNA content of the bacteria.

Protein synthesis continues long after the external level of a required purine or of the major carbon and energy source has been reduced to a value insufficient to permit RNA accumulation. This observation is quite compatible with the concept that the role of ribosomal RNA in protein synthesis is a passive one.

The most significant observation in this regard, however, concerns the transition from a slow to a rapid growth rate. The fact that the rate of protein synthesis does not attain its new high value until after the RNA level of the protoplasm has been increased to that characteristic of the new growth rate strengthens the argument for a passive role of ribosomal RNA in protein synthesis. Were it the rate of RNA synthesis *per se* which determines the rate of protein synthesis, then one would have expected as rapid an acceleration in the latter as in the former.

Turning now to the question of the control of RNA synthesis, we would like to suggest that the rate of RNA synthesis is more sensitive to the composition of the medium than is the rate of protein synthesis. It would appear that some metabolite whose intracellular concentration is raised by enrichment of the medium preferentially stimulates RNA synthesis. From the present experiments it is impossible to learn the nature of such a metabolite, for every compound which stimulates growth when added externally causes this marked stimulation of RNA synthesis, and all of them, of course, are interconvertible by the cell. One guess as to its nature, however, stems from the fact that the synthesis of RNA in the presence of chloramphenicol requires the availability to the cell of all of the amino acids[29,30]: activated amino acids or amino acid–nucleotide complexes might be the limiting factor in RNA synthesis.

Now if we assume that RNA synthesis is particularly sensitive to the level of some (unknown) constituent whose concentration depends on the metabolic activity of the cell, what would be the consequence of changes in the composition of the medium? A sudden drop in the available supply of carbon and of energy would temporarily diminish RNA synthesis to a greater extent that it would protein synthesis. Conversely, a sudden increase in the available supply of carbon and energy would stimulate RNA formation preferentially.

An important subject for further investigation is the discovery of the particular compound which is rate-limiting for RNA synthesis. But it is already possible with the present results to assess the significance of the control of protein and RNA synthesis to the economy of the cell. The fluctuations of RNA content in growing cells permit the cells to grow at a faster rate in any particular chemical environment than would be possible with an inflexible system. Thus, the cell growing on *myo*-inositol, which is not metabolized rapidly enough to give the maximal growth rate, uses a smaller proportion of its resources to make RNA than that used by a cell growing on glucose; hence, a larger portion of the energy and building blocks produced by the degradation of *myo*-inositol is available for the synthesis of the other components of the bacterial cell. With an inflexible system in which the protein-forming machinery were always present in maximal amount, the cell would grow with maximal efficiency only at its maximal growth rate.

The existence of this adjustment mechanism in bacterial metabolism is far from surprising: studies on the control of synthesis of individual enzymes in bacteria have shown these organisms to be quite economical in the handling of their resources. The same economy seems to apply in part at least to the production of RNA.

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REFERENCES

[1] B. MAGASANIK, A. K. MAGASANIK AND F. C. NEIDHARDT, in G. E. W. WOLSTENHOLME AND C. M. O'CONNER, *Regulation of Cell Metabolism* (CIBA Foundation Symposium), Little, Brown and Company, Boston, 1959.
[2] A. TISSIÈRES AND J. D. WATSON, *Nature*, 182 (1958) 778.
[3] R. LOFTFIELD, *Progress in Biophysics and Biophysical Chemistry*, Vol. XIII, Pergamon Press, London, 1958.
[4] J. BRACHET, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. II, Academic Press, Inc., New York, 1955.
[5] A. CAMPBELL, *Bacteriol. Rev.*, 21 (1957) 263.
[6] M. SCHAECHTER, O. MAALØE AND N. O. KJELDGAARD, *J. Gen. Microbiol.*, 19 (1958) 592.
[7] N. O. KJELDGAARD, O. MAALØE AND M. SCHAECHTER, *J. Gen. Microbiol.*, 19 (1958) 607.
[8] D. USHIBA AND B. MAGASANIK, *Proc. Soc. Exptl. Biol. Med.*, 80 (1953) 626.
[9] W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
[10] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[11] G. CERIOTTI, *J. Biol. Chem.*, 198 (1952) 297.
[12] K. KECK, *Arch. Biochem. Biophys.*, 63 (1956) 446.
[13] G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
[14] J. MANDELSTAM, *Biochem. J.*, 69 (1958) 110.
[15] B. MAGASANIK, *J. Biol. Chem.*, 205 (1953) 1019.
[16] F. C. NEIDHARDT AND B. MAGASANIK, *Biochim. Biophys. Acta*, 21 (1956) 324.
[17] F. C. NEIDHARDT, unpublished experiments.
[18] H. E. WADE AND D. M. MORGAN, *Biochem. J.*, 65 (1957) 321.
[19] A. D. HERSHEY, *J. Gen. Physiol.*, 38 (1954) 145.
[20] A. L. KOCH AND H. R. LEVY, *J. Biol. Chem.*, 217 (1955) 947.
[21] G. SCHMIDT, K. SERAIDARIAN, L. M. GREENBAUM, M. D. HICKEY AND S. J. THANNHAUSER, *Biochim. Biophys. Acta*, 20 (1956) 135.
[22] J. T. HOLDEN, *Biochim. Biophys. Acta*, 29 (1958) 667.
[23] J. MENDELSOHN AND A. TISSIÈRES, *Biochim. Biophys. Acta*, 35 (1959) 248.
[24] P. C. CALDWELL AND C. HINSHELWOOD, *J. Chem. Soc.* (1959) 3156.
[25] W. H. PRICE, *J. Gen. Physiol.*, 35 (1952) 741.
[26] H. E. WADE, *J. Gen. Physiol.*, 7 (1952) 24.
[27] R. JEENER, *Arch. Biochem. Biophys.*, 43 (1953) 381.
[28] F. H. C. CRICK, in *Biological Replication of Macromolecules*, Symposium XII of the Society for Experimental Biology, Academic Press, Inc., New York, 1958.
[29] A. B. PARDEE AND L. S. PRESTIDGE, *J. Bacteriol.*, 71 (1956) 677.
[30] F. GROS AND F. GROS, *Biochim. Biophys. Acta*, 22 (1956) 200.

THE INDUCTION OF MUTANTS OF BACTERIOPHAGE T2 BY 5-BROMOURACIL

III. NUTRITIONAL AND STRUCTURAL EVIDENCE REGARDING MUTAGENIC ACTION*

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SUMMARY

A study has been made of the induction of mutants of bacteriophage T2 by thymine analogues, in particular by 5-bromouracil (BrU) and 5-bromouracil deoxyriboside. BrU was mutagenic only when the host bacteria were subjected to thymine deficiency brought about in the present work by the addition of sulfanilamide. In media containing sulfanilamide and BrU, *E. coli* B exhibited an absolute requirement for uracil, which was eliminated upon phage infection. The induction of phage mutants by BrU was greatly influenced by the various nutrients provided in the medium. To obtain reproducible results, a defined medium was derived which contained salts, glucose, sulfanilamide, valine, methionine, xanthine, and uracil. In this medium three substances prevented BrU mutagenesis, adenine and serine partially and thymidine completely; these substances are involved in DNA synthesis.

BrU was incorporated into phage DNA in place of thymine in large amounts (nearly 100 % in two experiments). This incorporation was always accompanied by the appearance of a high proportion of mutants and a yet higher proportion of non-infective phage, which were also unable to kill *E. coli*. The infective population of phages grown in sulfa and BrU were more sensitive to u.v. light than were normal phages. Of the many pyrimidine analogues tested, only chloro-, bromo-, and iodo-uracil (not fluorouracil) and especially the deoxyriboside of BrU, exhibited mutagenic activity, which was in all cases specifically prevented by thymidine. All four of these compounds can substitute for thymine in phage DNA. The hypothesis is put forward that BrU mutagenesis is mediated through its incorporation into DNA, but that the incorporation *per se* is insufficient to cause the mutagenesis.

INTRODUCTION

At the present time, there are many lines of evidence which link the chemistry of the material carrying genetic information to the chemistry of deoxyribonucleic acid

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(DNA). To study the chemistry of mutations, therefore, use should be made of substances known to have a direct action on DNA. One class of such substances are the halouracil analogues of thymine, which have been shown to be incorporated into the DNA of several microorganisms[1-3]. We have observed that, when the coli-bacteriophage T2 was grown under conditions in which it could incorporate one of these analogues, 5-bromouracil (BrU) into its DNA, large numbers of mutants appeared among the progeny. The proof that these mutants arose as a result of mutagenic induction and not by selection of preexisting mutants and some preliminary experiments on BrU-induced mutagenesis have been reported in a previous publication[4]. It has furthermore been shown that protein synthesis is not essential for the initiation of the series of events leading to mutation by BrU[5,6].

Because of its direct connection with DNA, therefore, BrU would appear to be an ideal mutagenic agent to utilize for the study of the chemistry of genetic alterations. This paper is concerned with the demonstration of the direct connection between BrU-induced mutagenesis and thymine metabolism and with a description of the chemical and physiological properties of bacteriophages grown in the presence of BrU. The accompanying paper[7] presents the results of kinetic investigations aimed at the elucidation of the mechanism of the BrU-induced mutagenesis.

MATERIALS AND METHODS

Compounds

Amino acids: Two vitamin-free casein hydrolysates were employed in this study. The first, casein hydrolysate I, was prepared in this laboratory and contained principally aspartic acid, threonine, glycine, alanine, serine, glutamic acid, valine, leucine, tyrosine, and lysine. The second was purchased from Difco Laboratories and contained, in addition to the amino acids found in casein hydrolysate I, histidine and arginine. All of the individual L-amino acids were obtained commercially.

Purines: Adenine, guanine, xanthine, and hypoxanthine were all purchased from the Nutritional Biochemicals Corp. 6-methylaminopurine was kindly supplied by the Wellcome Research Laboratories, and all of the purine nucleosides by Dr. C. A. DEKKER.

Pyrimidines: Uracil, 5-aminouracil, and 5-bromouracil were purchased from the Nutritional Biochemicals Corp.; [2-^{14}C]uracil from the Research Specialities Co.; thymine and thiothymine from Dougherty Chemicals; 5-nitrouracil, 5-hydroxyuridine, 5-hydroxyuracil deoxyriboside, 5-bromouridine, 5-bromouracil deoxyriboside, and thymidine from the California Foundation for Biochemical Research. 5-diazouracil, 5-ethyluracil, 6-azauracil, and 6-azathymine were gifts from Dr. E. SASSENRATH; 5-iodouracil was a gift from Dr. P. NEWMARK and 5-fluorouracil from Hoffman-LaRoche Inc.

The 5-bromouracil (BrU) obtained commercially was found to contain a 5 % by weight impurity of uracil; pure BrU was synthesized by the method of WHEELER AND MERRIAM[8] from uracil and bromine. 5-chlorouracil was synthesized in this laboratory by the method of BARRETT, GOODMAN AND DITTMER[9].

Antibiotics: Sulfanilamide (sulfa) was purchased from Mallinckrodt Chemical Works.

Media

The basic synthetic medium used was the glucose–salts medium, M-9 (NH_4Cl, 1 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g, $MgSO_4 . 7H_2O$, 0.2 g; and glucose, 4 g/l of of water[10]. The various additions to this medium will be mentioned in the text, but sulfa when employed was present to the extent of 1 mg/ml. The defined medium developed experimentally, VM medium, contains per liter of M-9: sulfa, 1 g; xanthine, 10 mg; uracil, 2.5 mg; valine, 12 mg; and methionine, 9 mg. The glycerol-casamino acids medium of FRASER AND JERREL[11] was employed for the growth of seed cultures and for bacteria used for phage assays. Adsorption medium consisted of the following per liter of distilled water: NaCl, 4 g; K_2SO_4, 5 g; KH_2PO_4, 1.5 g; Na_2HPO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2$, 5.5 mg; and gelatin, 10 mg; it was used for making bacterial and phage dilutions.

Bacterial strains

Unless otherwise noted *E. coli* B will refer specifically to a substrain *E. coli* B (American), which was found to support a larger production of phage in sulfa containing media than any of the other *E. coli* B strains tested.

Bacteriophage strains

The T2r^+ and T2r_2 phage stocks were obtained from Dr. G. S. STENT. The T2r_2 stock employed throughout most of this work exhibited a 0.1 % background of plaque-type mutants. In general standard phage techniques as described by ADAMS[12] were employed. Phages were always plated on nutrient agar. When desired, high titer phage stocks were prepared according to the method of HERRIOTT AND BARLOW[10] and purified by differential centrifugation.

Experimental procedure for the growth of bacteria and bacteriophage

The incorporation of BrU into the DNA of bacteriophages occurs most efficiently when the bacteria to be infected have been subjected to thymine deficiency. Such conditions can be achieved either by the employment of thymine-requiring bacterial mutants[1,3] or by the addition of an inhibitor of thymine synthesis such as sulfa[2] to the growth medium of normally thymine sufficient organisms. Sulfa has been employed throughout this work, because it is applicable to various strains of *E. coli* and because it provides the opportunity for considerable variation in the experimental conditions. Sulfa reduced the infective phage yield about 10 times in VM medium.

In most experiments, an inoculum of *E. coli* B grown overnight in M-9 was added to the desired experimental medium (containing sulfa) to give a concentration of about $2 \cdot 10^7$ bacteria/ml. Growth with aeration at 37° for four generations was followed by infection with bacteriophage. This extent of growth was sufficient to establish a partial sulfa inhibition, but the resultant bacteria were completely viable and did not differ in appearance from those grown normally. Unless otherwise noted, the bacteria were always infected with a phage multiplicity of above one. Under these conditions lysis inhibition ensued, even with a T2*r* phage strain. The mature phages could be released by lysing the infected cells with chloroform.

The effects on phage will be reported in terms of infective phage yields and percentage of plaque-type mutants. (Only plaque-type mutants were scored in the present work.) The infective phage yield is the number of phage particles capable of forming plaques produced per infected bacterium, and the percentage of plaque-type mutants represents the number of mutant plaques observed × 100 divided by the total number of plaques. For each determination of the percentage of plaque-type mutants, at least 1,000 plaques were counted.

Determination of DNA base composition

For the determination of BrU incorporation into the DNA of bacteriophage T2, phage lysates were prepared in large volumes of various media. After four generations of growth, the bacteria were infected with a low phage multiplicity, and the infection allowed to proceed overnight. The resultant phage particles were purified by differential centrifugation and then hydrolysed with 88 % formic acid in a nitrogen atmosphere[13]. The DNA bases were separated by two dimensional chromatography on Whatman No. 1 paper, with isopropanol-HCl[14] and n-butanol-NH_3[15] as the first and second solvents, respectively. Thymine and BrU had the same R_f in the first dimension, but they were separated by the second solvent, thymine having an R_f of 0.44 and BrU of 0.35. The bases were eluted from the paper with 0.1 *N* HCl and their concentration determined from optical density readings.

Technique for u.v. irradiation

The phages to be irradiated were suspended in adsorption medium, 3 ml placed on a 10-cm watch glass and irradiated at a distance of 82 cm with 350 ergs/sec/cm² of u.v. from 0.35 amp. Rad-i-air lamp, whose main output was at 2537 Å. After each consecutive 8-sec irradiation, aliquots were withdrawn for later dilution and plating for phage survivors. The platings were performed in semidarkness to prevent photoreactivation. In each case, the experimental samples which were progeny of $T2r_2$ were admixed with approximately equal numbers of a control phage stock of a different plaque-type, $T2r^+$. Due to the presence of $T2r^+$ in the same irradiation vessel, the resultant u.v. inactivation curves could be corrected for the protection against u.v. afforded by the presence in the medium of any nucleic acid derivative.

RESULTS

Nutritional requirements for the sulfa and bromouracil system

The growth of *E. coli* in the presence of sulfa requires the addition of certain amino acids, a purine, and a pyrimidine[16]. In our[4] early studies of BrU-induced mutagenesis, the medium of DUNN AND SMITH which contains a vitamin-free casein hydrolysate, xanthine, and thymine, respectively was employed. The results obtained with one preparation of casein hydrolysate differed from those obtained with another, and the substitution of xanthine and thymine by other naturally occurring bases also caused variations in the number of phage and phage mutants produced. A series of investigations was therefore undertaken to determine which of the amino acids, purines and pyrimidines gave the greatest yield of mutants, to develop a simple defined medium that gave reproducible results, and to obtain information on the mechanism of mutagenesis.

Effects of various amino acids on mutagenesis: The amino acid requirements for mutant production and phage development were investigated by two methods: supplementation of a synthetic medium by individual amino acids, and amino acid elimination from a complete medium. In all of these experiments, the purine requirement was fulfilled by xanthine and the pyrimidine requirement by uracil.

In the tests of the effects of addition of individual amino acids, valine, methionine, and serine were first chosen for study because these three had been found by WINKLER AND DE HAAN[16] to support the growth of *E. coli* in a sulfa containing medium. It can be seen from Table I that T2 grew and was caused to mutate in the absence of added amino acids. Serine alone or in combination with the other amino acids stimulated T2 production, but greatly reduced the fraction of mutants. The highest yields of mutants and total phage were obtained in the presence of valine plus methionine. Addition of a few other amino acids, notably leucine, did support increases in infective phage production, but none significantly increased the mutagenic power of BrU. The above observations led to the synthetic medium (VM-medium, described under METHODS) which was used in most of the experiments to be reported. A number of variations of this medium were studied, but in general they showed little effect or were not beneficial to mutant production.

TABLE I

EFFECTS OF THE ADDITION OF VALINE, METHIONINE, AND SERINE ON PHAGE AND PHAGE MUTANT PRODUCTION

E. coli B was inoculated into M-9 medium supplemented with 1 mg/ml sulfa, 25 µg/ml xanthine, 50 µg/ml BrU, 2.5 µg/ml uracil, and the desired amino acid mixture. After four generations of growth, the bacteria were infected with a $T2r_2$ multiplicity of two, and 2–3 h later the infected cells were caused to lyse by the addition of chloroform. The progeny phages were then diluted and plated on *E. coli* B, and the results registered in terms of infective phage yield per bacterium and the percentage of plaque-type mutants.

Amino acid			*Infective phage yield*	*% Plaque-type mutants*
Serine (25 µg/ml)	*Valine (40 µg/ml)*	*Methionine (30 µg/ml)*		
—	—	—	50	2.5
+	—	—	87	1.1
+	+	—	130	1.2
+	—	+	87	1.0
+	+	+	150	1.1
—	+	—	45	3.8
—	—	+	30	1.9
—	+	+	100	3.9

Very similar results were obtained when, in VM medium, BrU was added at the time of phage infection instead of at the time of bacterial inoculation. With varying BrU concentrations the percentage of mutants rose to a maximum at 50 µg/ml BrU and then decreased at higher concentrations of BrU (Fig. 1).

At a multiplicity of 2 T2 per bacterium, BrU inhibited phage production by about 60% (Fig. 1); but at a multiplicity of 6 T2, the BrU was not inhibitory (see Table IV).

In all of these experiments, the phage yields were higher and the percentage of mutants somewhat lower than at the same BrU concentration in media rich in amino acids. This discrepancy was investigated by elimination of groups of amino acids from a complex medium. Results obtained with media containing Difco casein hydrolysate were not changed greatly when a mixture of 18 amino acids[17] was

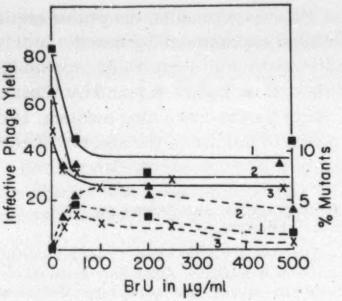

Fig. 1. Pattern of action of varying concentrations of BrU on phage and phage mutant production in valine and methionine media. Expt. 1: Valine 40 μg/ml and methionine 30 μg/ml. BrU added at time of bacterial inoculation. Expt. 2: Valine 12 μg/ml and methionine 9 μg/ml. BrU added at time of bacterial inoculation. Expt. 3: Valine 12 μg/ml and methionine 9 μg/ml. BrU added at time of phage infection. In all three experiments, the bacteria were infected with 2 phages per bacterium and lysed with chloroform 2 h later. ——, infective phage yield; – – –, percentage of plaque-type mutants.

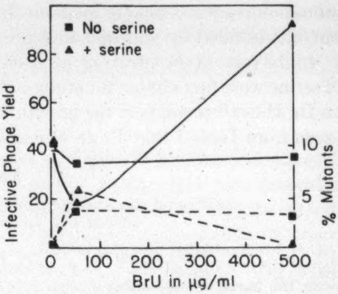

Fig. 2. Pattern of action of varying concentrations of BrU on phage and phage mutant production in 13 amino acid medium or in the same medium without serine. BrU added at time of bacterial inoculation. The bacteria were infected with 2 phages per bacterium and lysed with chloroform 2 h later. ——, infective phage yield; – – –, percentage of plaque-type mutants.

employed (or even with 7 amino acids including serine, although in this case only 3 % mutants appeared). Data obtained in a medium containing 13 amino acids, including serine, found by Fowler and Cohen[18] to support the growth the growth of T2 as satisfactorily as more complex media, gave results like those obtained with casein hydrolysate. Upon removal of serine the data took on a very different appearance (Fig. 2); and the results were now similar to those obtained with VM medium (Fig. 1). In the presence of serine a marked dependence of the percentage of mutants and phage yield on BrU concentration was noted, whereas in the absence of serine the results were relatively independent of BrU concentration.

An explanation of the partial prevention of the mutagenic action of BrU by serine in the simple VM medium (Table I), is that the β-carbon of serine is normally utilized for the synthesis of the 5-methyl group of thymine and the 5-hydroxymethyl group of 5-hydroxymethylcytosine of phage DNA[19] therefore, serine was undoubtedly utilized as a precursor for the synthesis of the natural phage pyrimidines, since the block of one carbon transfer reactions by sulfa was not complete. Thus the effect of the one amino acid which partially prevented the action of BrU can be related to the synthesis of thymine.

Purine supplementation and mutagenesis: In the experiments reported so far, the purine, xanthine, had always been added to the media; but the addition of other naturally occurring purines greatly affected the action of sulfa and BrU (Table II). The best combination of high phage yields and high percentages of mutants were obtained when xanthine was provided. All of the adenine compounds inhibited the production of phage and phage mutants and also bacterial growth when added singly or in combination with other purines. In the presence of purine nucleosides, the phage yields were always slightly lower with the ribose compounds than with the corresponding deoxyribonucleosides. 6-methylaminopurine, which increases in concentration under conditions of thymine deficiency[20], did not reverse bromodeoxyuridine action and probably did not function as a methyl donor, nor did it behave like an adenine derivative.

On the basis of the results of Table II, it would appear that the most desirable of the purines for the expression of mutagenesis by BrU is xanthine, and that adenine compounds counteract BrU action.

TABLE II

EFFECTS OF THE ADDITION OF PURINE COMPOUNDS ON THE PRODUCTION OF PHAGE AND PHAGE MUTANTS

E. coli B was inoculated into M-9 medium supplemented with the following per ml: 1 mg sulfa, 12 μg valine, 9 μg methionine, 2.5 μg uracil, and 0.65 μmoles of the desired purine. After four generations of growth, the bacteria were infected with a high multiplicity of T2r_2, and the infected cells caused to lyse by the addition of chloroform 2 and 3 h later for Expts. A and B, respectively. In Expt. A, 50 μg/ml BrU was added at the time of bacterial inoculation, and in Expt. B, 50 μg/ml bromodeoxyuridine at the time of phage infection.

Purine added	*Expt. A*		*Expt. B*	
	Infective phage yield	*% Mutants*	*Infective phage yield*	*% Mutants*
None	50	2.0	66	3.0
Xanthine	50	4.0	44	6.0
Hypoxanthine	25	3.0	25	6.5
Xanthine + hypoxanthine	23	1.5	—	—
Guanine	50	3.5	58	4.5
Guanine + xanthine	55	2.5	—	—
Guanine riboside	—	—	36	5.0
Guanine deoxyriboside	—	—	62	3.0
Adenine	30	2.0	28	1.5
Adenine + xanthine	10	1.0	—	—
Adenine + guanine	3	0	31	3.0
Adenine riboside	—	—	28	1.0
Adenine deoxyriboside	—	—	78	1.0

TABLE III

EFFECTS OF THE ADDITION OF PYRIMIDINES ON THE PRODUCTION OF PHAGE AND PHAGE MUTANTS

E. coli B was grown in VM medium for four generations and then infected with a high multiplicity of T2r_2. The pyrimidine compounds were all added at the time of phage infection, and the infected bacteria were all lysed by the addition of chloroform 2 h after infection.

Expt.	*Pyrimidine added*	*Pyrimidine concentration in μg/ml*	*Infective phage yield*	*% Plaque-type mutants*
A	None	—	57	0.1
	Thymine	50	73	0.1
	Thymidine	25	80	0.1
	BrU	50	57	1.9
	BrU + thymine	50 + 50	67	2.3
	BrU + thymidine	50 + 25	51	0.1
B	None	—	106	0.1
	BrUDR	50	39	4.5
	BrUDR + thymidine	50 + 25	91	0.1

Pyrimidine supplementation and mutagenesis: The *E. coli* B strain used in these experiments grew in VM medium in the absence of added thymidine or other pyrimidines, but upon the addition of BrU it exhibited an absolute requirement for uracil. The added uracil was shown by tracer techniques to be utilized for the synthesis of all of the pyrimidines of the RNA and DNA of the bacteria. This uracil requirement, however, disappeared upon phage infection. As uracil proved not to be inhibitory to phage development, it was left in the medium during the infectious process and was shown (using [2-^{14}C]uracil) to be utilized for the synthesis of the phage DNA pyrimidines.

Of the naturally-occurring pyrimidine compounds, only thymidine was able to prevent the mutagenic action of BrU (Table III). The specific reversal of the action of the BrU mutagenesis by thymidine, a compound not known to be utilized for anything other than DNA synthesis, provides strong evidence in support of a direct connection between BrU-induced mutagenesis and DNA metabolism.

Survey of pyrimidines for mutagenic action: A number of pyrimidine derivatives were tested for their ability to induce the appearance of plaque-type mutants of bacteriophage T2. The compounds exhibiting mutagenic action in VM medium were the 5-halouracil derivatives: 5-chlorouracil, 5-bromouracil, 5-iodouracil, and especially 5-bromouracil deoxyriboside (BrUDR) (Table IV) (see also[21]). BrUDR was both the most powerful mutagen and the most inhibitory to the production of infective phage. Even at a concentration as low as 0.02 μmoles/ml, BrUDR could induce the appearance of at least 1.5% mutants. Of the free halouracil bases, BrU induced the highest proportion of mutants, but a larger total number of mutants was obtained with chlorouracil. Iodouracil was the least effective mutagen of the three. None of the bases were inhibitory to phage growth and all are known to be incorporated into phage DNA in place of thymine[22]. Finally, the mutagenic action of all four compounds was prevented specifically by the simultaneous addition of thymidine.

TABLE IV

COMPARISON OF THE EFFECTS OF 5-HALOURACIL DERIVATIVES ON PHAGE AND PHAGE MUTANT PRODUCTION

E. coli B was grown in VM medium for four generations and then infected with 6 $T2r_2$ per bacterium. All of the 5-halouracil derivatives were added at the time of phage infection and at a concentration of 0.26 μmoles/ml. The infected bacteria were caused to lyse 3 h after infection by the addition of chloroform.

Halouracil	*Infective phage yield*	% *Plaque-type mutants*
None	115	0.1
5-chlorouracil	192	1.9
5-bromouracil	106	2.5
5-iodouracil	159	0.5
5-bromodeoxyuridine	33	7.7
5-bromouridine	73	0.4
5-fluorouracil	6	0.1

Bromouridine had a slight mutagenic effect (Table IV), but most likely it was converted by the infected bacteria into BrUDR, and the 0.4% mutants obtained was an expression of the extent of this conversion.

The halopyrimidine 5-F-uracil was not at all mutagenic and was very inhibitory to phage growth (see Table IV). Of the other compounds tested in VM medium the following had no demonstrable mutagenic action: uracil, 5-aminouracil, 5-diazouracil, 5-ethyluracil, 5-nitrouracil, 6-azauracil, 6-azathymine, 2-thiothymine, 5-hydroxyuridine, and 5-hydroxyuracil deoxyriboside. Most of these compounds did reduce phage yields.

Properties of bacteriophage grown in the presence of bromouracil

Incorporation of BrU into the DNA of T2: *Base ratios*: In agreement with DUNN AND SMITH[2], it was found that BrU affects the chemical composition of phage DNA only by replacing some of the thymine. Several examples of the molar base ratios of the DNA of T2r_2 grown in sulfa and BrU are presented in Table V and compared to the values obtained by WYATT AND COHEN[13] for normal T2*r* phages. Within experimental error, the ratios of adenine to guanine were normal, and the ratios of adenine to thymine + BrU were equivalent to the normal adenine to thymine ratio. The hydroxymethylcytosine values were always low, but as it is difficult to recover 100 % of this base, these low values probably were due to experimental losses and not to an effect of BrU. In the subsequent discussion, therefore, the per cent BrU incoporated will refer to BrU/(Thymine + BrU) × 100.

TABLE V

MOLAR BASE RATIOS OF T2r_2 PHAGES GROWN IN SULFA AND BROMOURACIL
Lysate numbers refer to preparations mentioned in Table VI.

Base	*Molar base ratios*							
	Lysate 6		*Lysate 7*		*Lysate 8*		*Normal T2r*[13]	
Guanine	1.06		1.15		1.05		1.09	
HMC*	0.78		0.85		0.87		1.03	
Adenine**	2.00		2.00		2.00		2.00	
Thymine	1.03	2.06	0.85	2.10	0.87	2.19	2.07	2.07
BrU	1.03		1.25		1.32		0.00	

* HMC = 5-hydroxymethylcytosine.

** Value assigned arbitrarily.

Extent of BrU incorporation and mutagenic action: A number of experiments designed to measure the amount of BrU incorporation into the DNA of T2, the per cent plaque-type mutants, and per cent infectivity of these phages are presented in Table VI. The growth media employed all had in common the presence of sulfa, xanthine, and uracil, but differed in the source of amino acids listed in the second column and in the BrU concentration listed in the third column. It can be seen that the actual amount of BrU incorporated was influenced by both the amino acid content and the BrU concentration in the medium. Sufficient data are not available to explain these differences.

Of the biological properties of the phages which had incorporated BrU, the most striking was that they all exhibited a high proportion of plaque-type mutants (listed in the fifth column of Table VI). There was no obvious relationship between the amount of thymine replaced by BrU and the percentage of plaque-type mutants produced. Even when almost all of the thymine was replaced by BrU, most of the

TABLE VI

INCORPORATION OF BrU INTO THE DNA OF BACTERIOPHAGE T2

Concentrations of amino acids: both casein hydrolysates, 1 mg/ml; methionine 30 μg/ml and valine 40 μg/ml.

Lysate No.	*Amino acid source*	*BrU in µg/ml*	*% BrU incorporated*	*% Plaque-type mutants*	*% infectivity*
1	Casein Hydrolysate I	50	100	11.5	9
2	Casein hydrolysate I	500	100	13	2
3*	Casein hydrolysate I	2000	67	11.5	24
4	Difco casein hydrolysate	50	54	11	6
5	Difco casein hydrolysate	50	55	8	6
6	Methionine + valine	25	50	4	53
7	Methionine + valine	50	60	7	54
8	Methionine + valine	50	60	6	39
9	Methionine + valine	200	78	7	30

* $T2r^+$; all other lysates started with $T2r_2$.

viable phages were not plaque-type mutants. This result would indicate that the incorporation of BrU alone does not explain the mutagenic property of this agent.

BrU incorporation and production of inactive phage: Another biological property affected by growth in the presence of BrU was the ability of the phage particles to form plaques. The infectivity of the purified lysates (listed in the last column of Table VI) was calculated from a comparison of the plaque titer and the total number of phages, as determined by the O.D. at 260 mμ. The phages grown with casein hydrolysate I (lysates 1 to 3) and with valine and methionine (lysates 6 to 9) exhibited an approximate reciprocity between BrU incorporation and phage infectivity (in agreement with the results of DUNN AND SMITH[2]). However, this relationship did not hold for the phage lysates grown with the Difco casein hydrolysate (lysates 4 and 5), in which the percentage of the phage population remaining infective was always smaller than the percentage of thymine in the phage. Therefore, phage lysates were partially inactivated by the incorporation of BrU into their DNA, but the extent of this inactivation was not always proportional to their content of BrU.

In the two preparations listed as having 100 % BrU incorporation and 9 and 2 % infectivity (lysates 1 and 2, respectively of Table VI) no thymine had been observed on the chromatograms of the DNA hydrolysates, but the ratios of adenine to BrU were 0.93 and 0.95, respectively. Normally, there is less adenine than thymine + thymine analogue (see Table V). It is quite possible, therefore, that a small quantity of thymine may have been overlooked. But even if the 100 % values were in error by 5 or 10 %, it would seem extremely unlikely that 90 to 95 % of the phage populations had incorporated all of the BrU while the remainder had none. Thus, although the results of lysates 1 and 2 are not absolutely conclusive, they do provide evidence that phages can be infective while containing BrU in their DNA.

Ability of non-infective phage to kill bacteria: A few studies were made to determine whether the non-infective phages could retain their ability to kill *E. coli* B. Varying numbers of phage were adsorbed to bacteria and the cultures were plated to determine bacterial colonies. From the fraction of surviving bacteria at any phage concentration, equal to e^{-M} according to the Poisson distribution formula, the multiplicity M of the killing particles can be calculated. The ratio of M to the known multiplicity of

TABLE VII

BACTERIAL KILLING ABILITY OF T2 GROWN IN SULFA AND BrU

E. coli B was grown to about 10^8 cells per ml in M-9 medium, then centrifuged and resuspended in the same volume of adsorption medium. This suspension was distributed into individual tubes, and kept on ice. Then serial dilutions of the phage lysates to be tested were added to the separate tubes after prewarming to 37°, and adsorption allowed to proceed for 10 min before dilution and plating for numbers of surviving bacteria. A was a purified lysate grown in BrU and shown to contain 98 % non-infective phage particles; B was a crude lysate.

Lysate	*Fraction of bacterial survivors* e^{-M}	*Multiplicity of killing particles* M	*Multiplicity of infective phage added* N	*Ratio of killing to infective particles* M/N
A	0.12	2.1	1.9	1.1
	0.225	1.5	1.1	1.35
	0.385	0.95	0.8	1.2
	0.61	0.45	0.4	1.1
	0.86	0.15	0.16	0.9
	0.96	0.05	0.08	0.6
B	0.00	—	3.0	—
	0.16	1.8	0.6	3.0
	0.76	0.27	0.13	2.1
	0.905	0.1	0.03	3.3
	0.95	0.05	0.013	3.9

infective, plaque-forming phages added, N, gives the number of killing particles present per infectious particle.

Two different results were obtained (Table VII) with these phage preparations, depending on their purity. In lysate A, a purified preparation of phages, the bacterial killing ability could be entirely accounted for by the infective phage population. This preparation was known to contain 98 % non-infective phages. Therefore, the phage particles rendered incapable of forming plaques had also lost the ability to kill bacteria. In lysate B there were about three times as many killing particles as infective phages. The phages were still suspended in their original growth media, and the excess in bacterial killing power must have been due to the presence of particles of non-viral or incomplete viral nature[23].

Sensitivity of phages to inactivation by u.v. light: In order to determine whether the infective phages resulting from growth in sulfa, or in sulfa and BrU, could be distinguished from infective phages grown in the absence of these compounds, their sensitivities to inactivation by u.v. light were measured. In each case, the experimental samples (T2r_2) to be irradiated were admixed with an approximately equal number of normal T2r^+ (see METHODS). With phages grown in sulfa alone (Fig. 3A), the initial shoulder always observed with T2 was greatly reduced, and, although the exponential part of the inactivation curve exhibited the same slope as the control, it extrapolated to only 1.4 times the initial titer rather than the normal 2 times. Thus sulfa grown phages were inactivated by u.v. light at the same rate as the control phages, but were more sensitive in terms of absolute survival at any one dose.

In Fig. 3B, the u.v. sensitivity of T2r_2 phages grown in the presence of sulfa and BrUDR is compared to that of normal T2r^+. The shape of the inactivation curve of the former is quite different from that of the control. The shoulder was diminished in sharpness as for the sulfa grown phages, but the straight part of the curve could be

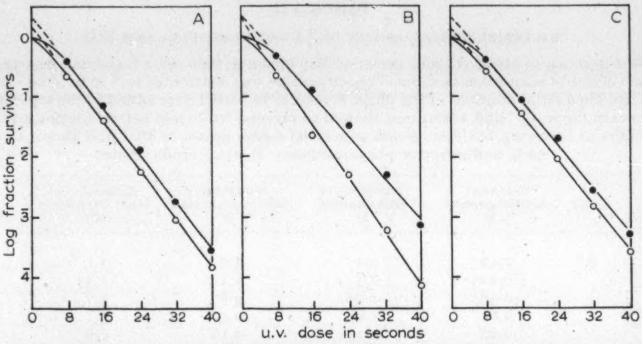

Fig. 3. u.v. inactivation curves of T2r_2 grown under various experimental conditions. A. T2r grown in sulfa alone. B. T2r_2 grown in sulfa and BrUDR. C. T2r_2 grown in sulfa, BrUDR and thymidine. ○ = experimental T2r_2; ● = normal T2r^+. Ordinate: log of the fraction of infective phage survivors.

extrapolated back to about twice the initial titer. The slope of this line, however, differed from that of T2r^+ by a factor of 1.27, indicating that the infective progeny of T2r_2 grown in sulfa and BrUDR are inactivated by u.v. light about 25 % more rapidly than are normal phage.

Thymidine was able to abolish the increased u.v. sensitivity brought about by growth in BrUDR. Fig. 3C presents the u.v. inactivation curve of phages grown in the presence of sulfa and both BrUDR and thymidine, and shows that thymidine prevented any change brought on by the mutagen. The shape of this curve was identical to that of sulfa grown phages (Fig. 3A), indicating once more that thymidine specifically reversed only the action of BrUDR and not the effect of sulfa (see Table III).

To determine whether BrU induced a heritable increase in u.v. sensitivity, an aliquot of phages grown in the presence of BrU was allowed to infect *E. coli* B in a normal synthetic medium, and the resultant progeny were tested for their reaction to u.v. These progeny no longer exhibited the u.v. sensitivity characteristics of their parents and their u.v. inactivation curve was identical in form to that of T2r^+.

On the basis of these observations one can state that the *viable* phages grown in sulfa and BrUDR (or BrU) are modified phenotypically in their structure. But it is not possible to decide whether the increased u.v. sensitivity of these phages is due to the presence of BrU in their DNA or to an alteration in the structure of other parts of the phage.

DISCUSSION

These studies have three objectives. The first is to provide a simple system for the study of BrU mutagenesis, in which one can reproducibly obtain an easily countable number of mutants. The second is to study the properties of phages containing BrU; and the third is to gather evidence from nutritional and analytical data regarding the manner in which BrU causes mutation. The first objective has been accomplished: a simple synthetic medium has been devised in which, under well-specified conditions, 5 to 10% plaque-type mutants can be routinely obtained in a single cycle of reproduction. It should be stressed that BrUDR, a mutagen superior to BrU, is needed to obtain this percentage of mutants. FREESE[21] has independently found BrUDR to be an excellent mutagen even in the absence of sulfa.

The principal conclusion regarding the nature of the mutagenic action is that reactions of thymidine are involved. The evidence for this view is several-fold: thymidine reverses the mutagenic action of BrU compounds completely; BrUDR, an anologue of thymidine, is the most effective mutagen; sulfa, which inhibits the synthesis of thymidine is required for the mutagenic action of BrU; serine which stimulates the synthesis of thymine decreases the mutagenic action of BrU.

The incorporation of BrU in place of thymine in DNA is most probably involved in the mutagenic action. With incorporation near 100% in some cases, it is likely that viable phages contain BrU—a suggestion supported by the finding that viable phages grown in the presence of BrU are more sensitive to u.v. light and to x-rays[24] than are normal phages. Perhaps the strongest piece of evidence in favor of this hypothesis is that only those pyrimidines that are incorporated in place of thymine (*e.g.*, the Cl-, Br-, and I-uracils) are mutagenic, although other pyrimidines have equally strong inhibitory effects on phage production.

The data presented here do not rule out an alternative hypothesis that a limited supply of thymine (caused by the combined action of sulfa and BrU) creates mutagenic alterations in the phage DNA; for example, substitutions or deletions could be brought about at sites normally occupied by thymine. Indeed, thymine deficiency has been shown to produce mutations in *E. coli*[25]. In the present experiments the lack of proportionality between the amount of BrU incorporated and the per cent mutants suggests that mutagenesis is not simply due to incorporation. Also, the nutritional complexities observed (effects of amino acids and adenine compounds and the *decrease* in the per cent mutants when high concentrations of BrU are added), and also the production of numerous inactive particles and lethal subunits in the presence of BrU suggest that the action of BrU is not restricted to its simple substitution into DNA in place of thymine; but whether these metabolic alterations have anything to do with the primary steps of mutagenesis is not clear.

Even though incorporation of BrU may be an essential step in the mutagenic process, it is not at all certain that this is the step at which the mutation is completed; *i.e.*, BrU in DNA might act in every way like thymine, and only when it is replaced by another base, perhaps HMC, would a hereditary change be established. Further investigations of these matters will be presented in the following paper[7].

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REFERENCES

[1] F. WEYGAND, A. WACKER AND H. DELLWEG, *Z. Naturforsch.*, 7b (1952) 19.
[2] D. B. DUNN AND J. D. SMITH, *Nature*, 174 (1954) 305; *Biochem. J.*, 67 (1957) 494.
[3] S. ZAMENHOF AND G. GRIBOFF, *Nature*, 174 (1954) 307.

[4] R. M. Litman and A. B. Pardee, *Nature*, 178 (1956) 529.
[5] S. Brenner and J. D. Smith, *Virology*, 8 (1959) 124.
[6] R. M. Litman and A. B. Pardee, *Virology*, 8 (1959) 125.
[7] R. M. Litman and A. B. Pardee, *Biochim. Biophys. Acta*, 42 (1960) 131.
[8] H. L. Wheeler and H. F. Merriam, *Am. Chem. J.*, 29 (1903) 478.
[9] H. W. Barrett, I. Goodman and K. Dittmer, *J. Am. Chem. Soc.*, 70 (1948) 1753.
[10] R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.*, 36 (1952) 17.
[11] D. Fraser and E. A. Jerrel, *J. Biol. Chem.*, 205 (1953) 291.
[12] M. H. Adams, *Methods in Med. Research*, 2 (1950) 1.
[13] G. R. Wyatt and S. S. Cohen, *Biochem. J.*, 55 (1953) 774.
[14] G. R. Wyatt, *Biochem. J.*, 48 (1951) 584.
[15] R. Markham and J. D. Smith, *Biochem. J.*, 45 (1949) 294.
[16] K. C. Winkler and P. G. de Haan, *Arch. Biochem.*, 18 (1948) 97.
[17] C. B. Fowler and S. S. Cohen, *J. Exptl. Med.*, 87 (1948) 259.
[18] C. B. Fowler and S. S. Cohen, *J. Exptl. Med.*, 87 (1948) 275.
[19] S. S. Cohen and L. L. Weed, *J. Biol. Chem.*, 209 (1954) 789.
[20] D. B. Dunn and J. D. Smith, *Nature*, 175 (1955) 336.
[21] E. Freese, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 622.
[22] R. E. F. Matthews and J. D. Smith, *Advances in Virus Research*, 3 (1955) 49.
[23] W. Weidel, *Ann. Rev. Microbiol.*, 12 (1958) 27.
[24] C. Steinberg, F. W. Stahl and M. Meselson, personal communication.
[25] C. A. Coughlin and E. A. Adelberg, *Nature*, 178 (1956) 531.

Biochim. Biophys. Acta, 42 (1960) 117–130

THE INDUCTION OF MUTANTS OF BACTERIOPHAGE T2 BY 5-BROMOURACIL

IV. KINETICS OF BROMOURACIL-INDUCED MUTAGENESIS*

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SUMMARY

A kinetic study has been made of the effects of 5-bromouracil deoxyriboside (BrUDR) on T2 development in a sulfanilamide-containing medium. As tested by the u.v. light sensitivity of the intracellularly developing phage and by the time of appearance of the first intracellular mature phages, it was found that sulfanilamide and BrUDR did not change the pattern of phage development, but slowed it down slightly. Sulfanilamide caused lysis inhibition of the r_2 phages in this medium. BrUDR did not begin to be effective as a mutagen until the time expected for commencement of phage DNA synthesis, but the mutagenic action of BrUDR could be exerted at any time thereafter as long as phage DNA continued to be synthesized. Counteraction of free BrUDR by thymidine at 15 min after infection did not halt the continued production of mutants. It is proposed that mutations are induced both in the process of the introduction of BrU into phage DNA and during the replication of DNA containing BrU. Two mechanisms by which BrU might induce the appearance of altered base sequences in phage DNA are outlined.

INTRODUCTION

The work presented in the preceding papers[1-3] points to a direct connection between bromouracil-induced mutagenesis and bacteriophage deoxyribonucleic acid (DNA) metabolism and composition. These studies were limited in that only the progeny phage and other final products were examined; that is, the results of certain treatments or conditions applied at the time of infection were examined only at the end of the phage developmental cycle. In the present communication, an attempt has been made to obtain information on the mechanism of bromouracil mutagenesis by a series of kinetic investigations concerned with the determination of when a bromouracil compound acts during phage development.

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The schedule of events occurring in T2-infected *Escherichia coli* B is now quite well defined. Although the subject of phage development has been treated at length in several recent reviews[4,5] the following brief discussion will suffice to permit correlation of the time of the establishment of the induced mutations with the development of phage. T2 infects *E. coli* B by first attaching by its tail to the host bacterium and dissolving a small region of the bacterial cell wall[6]. Then the phage DNA passes into the cell, while the phage protein coat remains on the surface of the bacterium and has no further function in phage reproduction[7]. Immediately following phage DNA entry, a reorganization of the metabolism of the bacterial host takes place. The synthesis of ribonucleic acid is reduced to 2 to 3% of that in the uninfected bacterium[8], total protein synthesis continues without interruption[9], the synthesis of bacterial DNA is at once stopped, and phage DNA synthesis starts at about 7 min[10]. Only phage DNA is synthesized in infected bacteria[11], and it continues to be synthesized throughout the latent period and is always present in excess of the number of mature phage particles formed[12]. During the first 12 min after infection, the phage has lost its identity as an infectious unit[13]. After 12 min, however, mature infective phages begin to make their appearance within the cell and continue to increase in number until the infected bacterium lyses at the end of the latent period[14].

MATERIAL AND METHODS

The materials and methods described in the preceding publications[2,3] were applied in the present work. 5-bromouracil deoxyriboside (BrUDR) was used as a mutagenic agent, since it caused many more mutants to appear than did 5-bromouracil[3]. The infective phage yield is the number of phage particles capable of forming plaques produced per infected bacterium, and the percentage of plaque-type mutants is the number of mutant plaques of all types observed × 100 divided by the total number of plaques.

RESULTS

Determination of the time course of T2 development in the presence of sulfanilamide and BrUDR

Since the correlation of the time course of the mutagenic action of BrUDR with other events in the infected bacterium was the principal objective of the present work, it was necessary to determine whether BrUDR seriously altered the schedule of phage development. Release of phages from infected bacteria with chloroform at various times after infection showed that in the presence of BrUDR the first mature phages appeared at about 15 min. This result suggests that phage development in the presence of sulfanilamide (sulfa) and BrUDR is slightly slower than in the absence of these compounds. (In the latter case, the first intracellular phage appear at about 12 min.) This conclusion is borne out by the experiments described below.

A second method of measurement of the time of appearance of the first intracellular phage was performed by plating with streptomycin resistant bacteria on streptomycin plates. Streptomycin stops phage development within 2 or 3 min after its addition, but the infected bacteria lyse at the normal time[15]. Therefore, until the appearance of the first mature phages, only the unadsorbed phages will form plaques on the streptomycin plates. The results of these platings are presented in Table I. It can be seen that the number of unadsorbed phages decreased slightly between 0 and 10 min, but by 15 min the plaque titer had begun to rise, indicating the appearance of mature phage before 18 min (15 min plus 3 min for the delay needed for streptomycin action). By extrapolation, one finds that the first mature phages made their appearance at about 15 min after infection. Under normal conditions, the first intracellular mature phages begin to appear at 12 to 13 min[13]. These results demonstrate once more that the time course of T2 development in the presence of sulfa and BrUDR was slightly delayed.

TABLE I

TIME OF APPEARANCE OF INTRACELLULAR PHAGE IN THE PRESENCE OF SULFA AND BrUDR

E. coli B was grown for four generations in 10 ml of VM medium[3] to a density of 10^8 cells per ml, then centrifuged, washed and resuspended in 1 ml of M-9 medium without glucose. T2r_2 was added at a multiplicity of 0.02 phages per bacterium, and adsorption allowed to proceed for 5 min at 37°. The infected culture was then diluted by the addition of 9 ml of the original growth medium now supplemented with 50 µg/ml BrUDR, and the time of dilution taken to be time 0 (90% of the phages had been absorbed by this time). At 0, 5, 10, 15, and 20 min after infection (and after the addition of BrUDR), aliquots of the infected bacteria were diluted and plated with a T2-sensitive, streptomycin resistant strain of *E. coli* B on agar containing 2 mg/ml of streptomycin.

Time after infection and addition of BrUDR in minutes	*Results of the streptomycin platings*		
	Plaque titer	*% Infected bacteria containing mature phage**	*Presence of plaque-type mutants*
0	$4.8 \cdot 10^3$	0	—
5	$3.0 \cdot 10^3$	0	—
10	$2.7 \cdot 10^3$	0	—
15	$5.6 \cdot 10^3$	15	+
20	$15.0 \cdot 10^3$	65	+

* = 100 × plaque titer at any time minus the number of unadsorbed phage divided by the number of originally infected bacteria per ml.

Table I also indicates the presence or absence of plaque-type mutants. Their exact proportion could not be determined because of the poor plating conditions obtained when using the streptomycin technique. Nevertheless, mutant plaques appeared only on the 15- and 20-min plates, those which exhibited plaques formed by infected bacteria containing mature phage. Therefore, mutants made their appearance at the same time as the first few intracellular mature phages.

To compare the time course of T2 development in the presence of sulfa and BrUDR with that occurring in infected bacteria in normal media, the sensitivity of infected bacteria to u.v. light at different times after infection was determined. In 1947, LURIA AND LATARJET[16] demonstrated that the u.v. sensitivity of the infected bacterial cell at different stages in the phage life cycle followed a very specific pattern and suggested that this pattern reflected the evolution of the infectious process. The technical improvements devised by BENZER[17] and by SYMONDS[15] were employed in the present experiments. The family of curves obtained (Fig. 1) closely resembles that obtained under normal conditions. At 0 min the resistance of the infective centers to u.v. light was slightly greater than that of free phage. The resistance to u.v. light increased slowly between 0 and 5 min, then very rapidly between 5 and 10 min, and finally reached a peak at 15 min after infection. At 15 min the resistance to u.v. light was about 30 times greater than that at 0 min. After 15 min, the resistance of the infective centers to u.v. light decreased. While the shapes of the curves presented in Fig. 1 and the extent of increase in u.v. light resistance were identical to those obtained normally, the results show that the development of the resistance of infected bacteria to u.v. light was slowed down by sulfa and BrUDR. Normally the peak of u.v. light resistance is obtained at 10 min, whereas in the present experiment, it occurred at about 15 min. Therefore, as determined by sensitivity to u.v. light, the development of T2 in the presence of sulfa and BrUDR folowed the normal pattern but was slightly retarded.

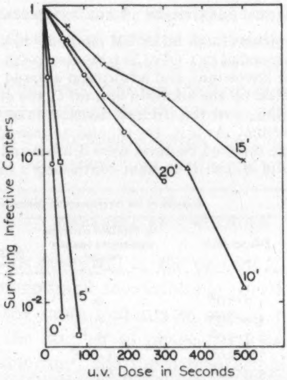

Fig. 1. Inactivation by u.v. light of infectious centers of $T2r_2$ in sulfa and BrUDR. The procedures for growth and infection of the bacteria were identical to that of the experiment of Table I. At 0, 5, 10, 15 and 20 min an aliquot was withdrawn, diluted 100-fold into ice-cold minimal salts buffer, and kept on ice until all of the samples for irradiation had been collected. 3 ml of each of these dilutions was placed on a separate watch glass over cracked ice and irradiated with 350 ergs/cm^2/sec of u.v. light from a 0.35 amp. Rad-i-air lamp at a distance of 82 cm for the lengths of time indicated in Fig. 1. After each dose of u.v. light, aliquots were withdrawn from the 3 ml and diluted and plated on *E. coli* B for numbers of surviving infective centers. The number on each curve gives the time in minutes between infection and dilution into cold buffer. Ordinate: fraction of infected bacteria which retained the ability to liberate phage.

Kinetics of phage and phage mutant production in the continuous presence of BrUDR

Since the time course of T2 development in the presence of sulfa and BrUDR follows the normal pattern at a slower rate, it is possible to determine at what stage during phage infection BrUDR exerts its mutagenic effects. First, studies were made of the phage and phage mutant production as a function of time by bacteria infected and allowed to remain in the presence of BrUDR. The results of the platings on *E. coli* B are plotted in Fig. 2 as a function of time after infection. The infective phage production in the chloroform treated aliquots proceeded at a nearly constant rate during the 180 min of the experiment. More striking, the number of mutants increased even more rapidly, so that the *percentage* of mutants actually rose with increasing time of exposure to BrUDR. At first this value rose very steeply, then more slowly, reaching a final value of 9 % at 180 min. Apparently few phages were released through spontaneous lysis (centrifuged samples), but among these the percentage of mutants also increased with time after infection and BrUDR addition. The results of this experiment show first that the infection of *E. coli* B with a high multiplicity of T2r_2 in the presence of sulfa and BrUDR maintained the bacteria in a lysis inhibited state, a phenomenon observed normally only with r^+ phages[18]; other experiments showed that BrUDR was not needed to produce lysis inhibition. Second, in this condition, the infected bacteria synthesized infective phages at a nearly constant rate; and third, the continued presence of BrUDR during infection caused the induction of a constantly increasing *proportion* of mutants. It would, therefore, appear that the mutagenic action of BrUDR continued to be expressed throughout the time that phages were being synthesized.

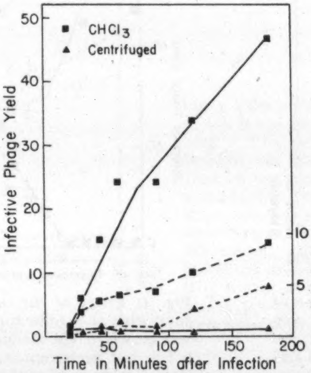

Fig. 2. Production of phage and phage mutants by bacteria in the presence of sulfa and BrUDR. *E. coli* B was grown in VM medium, infected with a multiplicity of 6 T2r_2 phages per bacterium, and 50 μg/ml BrUDR added immediately afterwards. At various times after infection, an aliquot of the infection mixture was lysed with chloroform to recover total mature phages, while another aliquot was centrifuged for 5 min at low speeds to remove unlysed bacteria and the supernatant fluid was analysed for phages released through spontaneous lysis. ■ = total mature infective phages; ▲ = infective phage released through spontaneous lysis. ——— = infective phage yield; - - - = percentage of plaque-type mutants.

To provide further support for the above conclusion, an experiment was performed to determine whether BrUDR could still induce the appearance of mutants if added at a time (40 min after infection) when the phage-producing mechanism was already established. The results obtained are plotted in Fig. 3. As was observed previously, in the 0′ sample, phages were produced at a linear rate, and the percentage of mutants rose with increasing time of exposure to BrUDR. In the culture to which BrUDR had been added at 40 min the rate of infective phage production, at first much greater than in the 0′ sample, was later diminished; but mutants appeared although only after a lag of a least 50 min. In the culture to which BrUDR was added at 40 min 5 % of the phages produced between 90 and 180 min were mutants. These data show that BrUDR must not cause mutant production only by modifying the initial steps of infection.

Effect of the removal of BrUDR at various times after infection

The results of the two preceding experiments suggest that the mutagenic action of BrUDR is not confined to any one period of phage development and can still be exerted during the late stages of phage reproduction. However, because the major reorganization of the metabolism of the infected bacteria takes place during the first few minutes after infection, it was of interest to determine whether the initial time of establishment of BrU mutagenesis could be correlated with a particular time during the initial stages of phage development. Experiments were performed to determine the effects of a brief initial exposure to BrUDR.

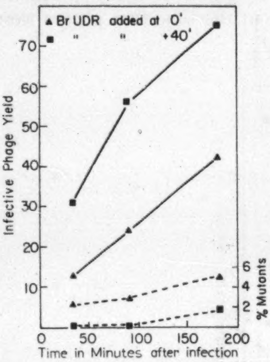

Fig. 3. Effects of the addition of BrUDR at infection or at 40 min afterwards. Two cultures of *E. coli* B were grown in VM medium and infected with 6 $T2r_2$ per bacterium. To one culture 50 μg/ml BrUDR was added immediately after infection (0′), while the other received 50 μg/ml BrUDR 40 min later (+40′). Aliquots were withdrawn from each culture at 35, 90, and 180 min to be lysed with chloroform. ——, infective phage yield; – – –, percentage of plaque-type mutants.

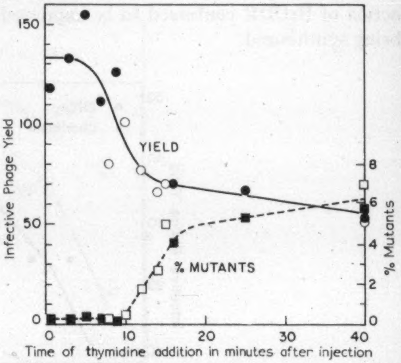

Fig. 4. Effect of the addition of BrUDR and of thymidine at various times after infection. *E. coli* B was grown in VM medium, infected at time 0 with 6 $T2r_2$ per bacterium, and 50 μg/ml BrUDR added immediately to all of the samples. Then at the times indicated in Fig. 4, 50 μg/ml thymidine was added to individual tubes and the infected bacteria in every sample lysed with chloroform three hours after infection. ——, infective phage yield; – – –, percentage of plaque-type mutants. Solid and open symbols distinguish the two experiments.

It was shown in the preceding paper[3] that thymidine, added simultaneously with BrUDR at the time of phage infection, had the property of specifically preventing the action of BrUDR; and it was found that the addition of this natural antagonist was a more effective way to stop BrUDR action than was simple dilution. The prevention of BrUDR action was studied as a function of time of thymidine addition. The results of two such experiments, plotted in Fig. 4 as a function of the time of addition of thymidine, represent the infective phage yield and the percentage of mutants obtained at the end of 3 h. If thymidine was added prior to 10 min after infection (and after the addition of BrUDR), the percentage of mutants obtained at 3 h remained at a constant low value, while the phage yields were quite high. This percentage of mutants and the phage yields were identical to those obtained in the sulfa control (not indicated in the Figure). Initially, therefore, the addition of thymidine effectively blocked the mutagenic and inhibitory action of BrUDR. But after this period the addition of thymidine became progressively less effective; the percentage of mutants began to rise and the phage yields to drop. The results also indicate that BrUDR is more effective during the period of 10 to 20 min after infection than it is later on.

It would appear, therefore, that BrUDR was effective as a mutagen only from about 10 min after infection on. This time coincides in the sulfa and BrUDR medium with the expected time of the start of phage DNA synthesis in sulfa and BrUDR medium, in which phage development is slowed down by about 3 min. The coincidence between the time of the effectiveness of BrUDR as a mutagen and the time of phage DNA synthesis suggests a requirement of DNA synthesis for the mutational event induced by BrUDR.

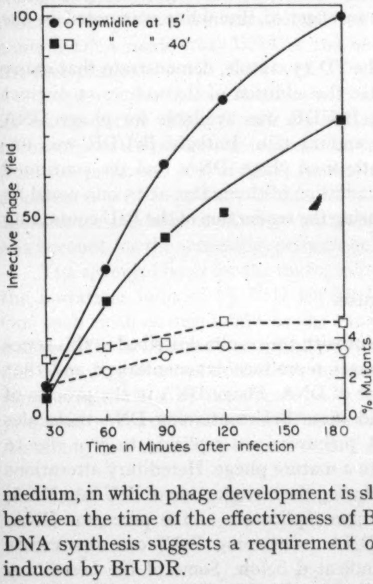

Fig. 5. Effects of an initial exposure to BrUDR. *E. coli* B was grown in VM medium, infected with 6 T2r_2 per bacterium, and 50 µg/ml BrUDR was added immediately afterwards. Then in one sample, 50 µg/ml of thymidine was added at 15 min (TD 15) and in another at 40 min (TD 40). At various times, aliquots were removed from both samples, lysed with chloroform, and diluted and plated. (The procedure used in this experiment was identical to that of Fig. 2 except for the addition in the present case of thymidine.) ——, infective phage yield; – – –, percentage of plaque-type mutants.

The technique utilized in the preceding experiment also presents an opportunity of learning when the mutations induced by BrUDR take place. As seen from Fig. 4, when thymidine was added as early as 15 min after infection (and after the addition of BrUDR) a high proportion (3.5 %) of mutants was observed when the bacteria were caused to lyse almost 3 h later. Does this percentage of mutants represent the extent of BrUDR mutagenic action between 10 and 15 min alone, or can changes produced during this time lead to new mutational events between 15 min and 3 h? In order to distinguish between these two possibilities, the rates of phage and phage mutant production were determined when BrUDR was added at the time of infection and thymidine subsequently at 15 (TD 15) or 40 min (TD 40).

The results plotted as infective phage yield and percentage of mutants as a function of time after infection are presented in Fig. 5. At all times the phage yield was higher and the percentage of mutants lower in the TD 15 sample than in the TD 40 sample. The total number of mutants increased at least 15-fold between 30 and 180 min in both cases. The percentage of mutants in both samples also rose for at least 90 min before attaining a final value of 3.5 and 5% for the TD 15 and the TD 40 samples, respectively, and was always higher than that existing at the time of thymidine addition.

In considering the interpretation of these data the possibility should be considered that thymidine might not immediately stop the action of free BrUDR. This is unlikely because at 30 min after infection, the TD 15 sample contained a lower percentage of mutants and a slightly higher phage yield than did the TD 40 sample to which thymidine had not yet been added. Although this does not prove that thymidine, immediately upon its addition, blocks BrUDR action, it does negate the possibility of a long delay in thymidine action, since an effect of thymidine was noted in the first aliquots taken after its addition.

The results of Fig. 5, at least those of the TD 15 sample, demonstrate that many new mutational events had taken place after the addition of thymidine, as distinct from those which had occurred when free BrUDR was available for phage DNA synthesis; *i.e.*, between approximately 10 and 15 min. Initially BrUDR was undoubtedly incorporated into the newly synthesized phage DNA, and the continued increase in the number of mutants after the addition of thymidine at 15 min could be due to new mutational events which arose during the replication of the BrU containing DNA.

DISCUSSION

The following model of BrU action is consistent with the results described in this series of papers. One assumes that vegetative phages reproduce exponentially[19] and that replicating units of vegatative phages consist of DNA. Phage DNA in the process of being synthesized can incorporate BrU, and these BrU-containing DNA molecules enter the pool of phage precursor DNA. A precursor can replicate to give rise to further precursors until it is incorporated into a mature phage. Hereditary alterations in the DNA structure can occasionally arise during the introduction of BrU into DNA, and these "mutations" can be replicated upon replication of the precursor DNA. Furthermore, replication of non-mutant DNA (containing BrU) can also create hereditary alterations by the mechanism indicated below. Some of the hereditary alterations in DNA structure are expressed as mutations in succeeding phage generations.

This scheme is consistent with the following observations, made in the present series of papers: (a) BrU incorporation is always accompanied by the appearance of mutants[3]. (b) BrU mutagenesis is prevented by thymidine and involves DNA synthesis[3]. (c) BrUDR mutagenesis commences at the same time as does phage DNA synthesis. (d) BrUDR is mutagenic in the absence of protein synthesis[2,20]. (e) The phages first formed include mutants. (f) A single bacterium releases both mutant and non-mutant phages[1]. (g) When BrUDR is present throughout the growth cycle, the number of mutants increases faster than the total number of phages; and therefore the percentage of mutants continually increases. (h) When the BrUDR is displaced by thymidine soon after mutagenesis is initiated, mutants continue to appear as an increasing percentage of the phages. (i) When BrUDR is added after some mature phages have already been formed, the percentage of mutants in the phages completed after that time gradually increases and eventually reaches a value similar to that found when BrUDR is added initially.

The validity of other schemes of mutagenesis should be considered in the light of the above observations.

According to the above scheme, the appearance of an increasing percentage of mutants with time is attributed to changes occurring on replication of the non-mutated, BrU-containing DNA. Alternative explanations can be advanced which depend on gradual enrichment of the phage precursor pool with mutated DNA, as the non-mutated DNA is withdrawn. (That the composition of the precursor pool can change is indicated by the experiment in which addition of BrUDR at 40 min causes mutants to appear.) One can imagine, for example, that since the first phages formed contain DNA made from DNA of the bacteria[21], fewer mutants would be found in the first phages.

Similarly, in the experiments in which BrUDR was effectively present only for an initial period (Fig. 5) and yet mutants continued to appear as an increasing percentage of the phages, an explanation can be devised based on a gradual maturation from the precursor pool of DNA synthesized in the presence of BrUDR. However, a calculation of the quantity of this initial DNA suggests that it is far too small to account for the large number of mutants eventually produced: nor does this hypothesis account for the increasing percentage of mutants with time.

The chemical basis for the mutagenic action of BrU remains uncertain. However, the mutations induced by BrU appear to involve very small molecular changes[22]. One such small change might be the substitution of one base pair for another in the double-helix DNA structure, which would lead to an altered base sequence. (This also has been suggested as a basis for mutation by FREESE[23].) The appearance of spontaneous mutations by the chance occurrence of altered base sequence was originally proposed by WATSON AND CRICK[24] as a corollary to their double-stranded DNA model. Assuming that the incorporation of BrU is a necessary prerequisite for its mutagenic action, how might this incorporation lead to changes in the base sequence of phage DNA? In the WATSON AND CRICK DNA structure BrU should, with high probability, pair with adenine to form hydrogen bonds identical to those between thymine and adenine. But BrU might have a small probability of pairing with guanine; though this probability could be great as compared to the probability of thymine pairing with guanine. A hydrogen bonded BrU-guanine pair is pictured (Fig. 6) which assumes that BrU is more stable in the enol form than is thymine (though at the present time there is no evidence to support this assumption). A mutant would be created if BrU was incorporated into DNA opposite guanine and on the next replication adenine was

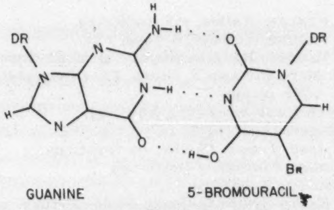

Fig. 6.

incorporated opposite BrU. On the other hand, if BrU was first incorporated opposite adenine and on the next replication guanine was incorporated opposite BrU a mutation would also be created. These events would give rise to mutants of different types, since in the former case the final product is the substitution of a guanine-hydroxymethylcystosine pair by an adenine-thymine pair, whereas in the latter case the opposite will be obtained.

The attractiveness of the above scheme is that it is in agreement with the idea that BrU-induced mutations consist of very small chemical changes, and that mutations can appear after free BrUDR is removed. The exchange of one set of base pairs would be sufficient to cause a mutation and the overall chemical composition of a phage lysate containing even a large proportion of mutants need not be detectably altered. It also helps to explain why only about 10 % mutants are obtained even when almost all of the thymines in DNA are replaced by BrU. However, BENZER AND FREESE[22] have reported the striking fact that BrU does not have a random mutagenic effect but induces certain mutations and back mutations[23] much more frequently than others. Therefore, the induction of mutations by BrU cannot be a matter of simple probability of pairing with guanine, but must depend also on the position in the phage genome into which the BrU is incorporated, either because some positions are more susceptible to permanent structure alterations than others or because only a limited number of these alterations cause non-lethal phenotypic effects.

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REFERENCES

[1] R. M. LITMAN AND A. B. PARDEE, *Nature*, 178 (1956) 529.
[2] R. M. LITMAN AND A. B. PARDEE, *Virology*, 8 (1959) 125.
[3] R. M. LITMAN AND A. B. PARDEE, *Biochim. Biophys. Acta*, 42 (1960) 117.
[4] R. M. HERRIOTT, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, Baltimore, The Johns Hopkins Press, 1957, p. 399.
[5] A. D. HERSHEY, *Advances in Virus Research*, 4 (1957) 25.
[6] W. WEIDEL, *Ann. Rev. Microbiol.*, 12 (1958) 27.
[7] A. D. HERSHEY AND M. CHASE, *J. Gen. Physiol.*, 36 (1952) 39.
[8] E. VOLKIN AND L. ASTRACHAN, *Virology*, 2 (1956) 149.
[9] S. S. COHEN, *J. Biol. Chem.*, 174 (1948) 295.
[10] S. S. COHEN, *Cold Spring Harbor Symposia Quant. Biol.*, 12 (1947) 35.
[11] G. A. VIDAVER AND L. M. KOZLOFF, *J. Biol. Chem.*, 225 (1957) 335.
[12] A. D. HERSHEY, *J. Gen. Physiol.*, 37 (1953) 1.
[13] A. H. DOERMANN, *J. Gen. Physiol.*, 35 (1952) 645.
[14] M. W. BENTZON, O. MAALØE AND G. RASCH, *Acta Pathol. Microbiol. Scand.*, 30 (1951) 243.
[15] N. SYMONDS, *Virology*, 3 (1957) 485.
[16] S. E. LURIA AND R. LATARJET, *J. Bacteriol.*, 53 (1947) 149.
[17] S. BENZER, *J. Bacteriol.*, 63 (1952) 59.
[18] A. H. DOERMANN, *J. Bacteriol.*, 55 (1948) 257.
[19] S. E. LURIA, *Cold Spring Harbor Symposia Quant. Biol.*, 16 (1951) 463.
[20] S. BRENNER AND J. D. SMITH, *Virology*, 8 (1959) 124.
[21] L. L. WEED AND S. S. COHEN, *J. Biol. Chem.*, 192 (1951) 693.
[22] S. BENZER AND E. FREESE, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 112.
[23] E. FREESE, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 622.
[24] J. D. WATSON AND F. H. C. CRICK, *Cold Spring Harbor Symposia Quant. Biol.*, 18 (1953) 123.

STUDIES ON MUCOPROTEINS

II. ANALYSIS OF THE PROTEIN MOIETY OF OVINE SUBMAXILLARY GLAND MUCOPROTEIN

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SUMMARY

Ovine submaxillary gland mucoprotein (OSM), shown previously to have the disaccharide α-D-N-acetylneuraminyl (2→6) N-acetylgalactosamine as prosthetic group, was analysed for its constituent amino acids and its amide content. OSM was found to have an almost complete complement of amino acids though with characteristic features. Its amide-N (0.34 % of total N) could only be determined after enzymic removal of its terminal neuraminic acid residues since even on mild acid hydrolysis the release of amide-N was accompanied by the degradation of N-acetylneuraminic acid with liberation of about half its N as ammonia-N. OSM exhibited a single peak on electrophoretic examination and in the ultracentrifuge.

INTRODUCTION

In the biological test ovine submaxillary gland mucoprotein (OSM) is the most potent of the influenza virus haemagglutinin inhibitory mucoproteins[1]. Chemically OSM is characterized by the presence in it of equimolecular quantities of N-acetylneuraminic acid (NANA) and N-acetylgalactosamine (NAGal) with traces only of other sugars, the latter being regarded as carbohydrate moiety of a contaminating mucoprotein. In a previous paper[2] the isolation, purification and analysis of the prosthetic group was described and its structure shown to be that of the disaccharide 6-α-D-N-acetylneuraminyl-NAGal. The carbohydrate-prosthetic group constitutes 42 % by weight of the mucoprotein molecule.

It seemed desirable to have a qualitative and quantitative analysis of the protein moiety of OSM including its amide content since no complete protein analysis is available for any of the influenza virus haemagglutinin inhibitory mucoproteins. Such an analysis was also required for a more detailed investigation into the type of linkage joining the prosthetic group to the polypeptide chain. Though CURTAIN AND PYE[3] have reported electrophoretic homogeneity for their OSM preparation, a comparison of their material with our OSM is not possible since the former authors have not characterized chemically their preparation. The homogeneity of OSM used in our work was therefore tested by electrophoresis and in the ultracentrifuge.

EXPERIMENTAL

Materials

OSM was prepared by the procedure described previously[2].

The amino acids used as reference substances were crystalline and of analytical grade.

The crystalline D-galactosamine hydrochloride was the preparation described previously[2].

Vibrio cholerae neuraminidase (RDE) was prepared in a highly purified state by Drs. G. ADA and E. FRENCH, Walter and Eliza Hall Institute of Medical Research, Melbourne, and kindly presented by them.

Ion exchange resins used were Dowex I-X2 (50–100 mesh) and Dowex 50-X8; the latter was hydraulically classified by the method of HAMILTON[4], particles 35–70 μ being selected.

Methods

50 mg samples of lyophilised OSM were hydrolysed for 20 and 70 h respectively with 5 ml of twice distilled constant boiling point hydrochloric acid in a sealed tube at 110°. The amino acids in the hydrolysate were separated by ion-exchange chromatography[5] and assayed with the ninhydrin reagent of MOORE AND STEIN[6]. The complete quantitation was carried out in the eight column automatic amino acid analysis equipment described by SIMMONDS AND ROWLANDS[7].

The total dicarboxylic acids in the hydrolysate of OSM (10 *N* HCl, 110°, 24 h) were determined independently by adsorption onto Dowex I-X2 (acetate form) followed by washing with water to remove neutral and basic amino acids and by eluting with acetic acid. The effluent and the eluate were separately lyophilised. The absence of aspartic and glutamic acids in the effluent and their presence uncontaminated by other amino acids in the eluate were verified by paper partition chromatography. The monoaminodicarboxylic acids in the eluate were determined according to MOORE AND STEIN[6].

Total nitrogen of OSM and of the ammonia-N in the 6 *N* HCl hydrolysate after 20 h and 70 h were determined by the micro Kjeldahl method.

Proline was determined in the acid hydrolysate of OSM by paper chromatography using serial dilutions of proline as standards and the isatin reagent[8] as spray; the solvent system was *n*-butanol–pyridine–water (6:4:3, v/v).

Alkaline hydrolysates of OSM (2 *N* NaOH or *N* $Ba(OH)_2$, 100°, 24 h) were tested for tryptophan by paper chromatography using the *p*-dimethylaminobenzaldehyde reagent as a spray.

Ammonia liberated from OSM by 2 *N* and *N* HCl respectively in a sealed tube at 100° for specified times was determined by the micro diffusion method of CONWAY AND O'MALLEY[9].

Total sulphur was determined according to ZIMMERMANN[10].

Electrophoresis was carried out at 2° in a Tiselius apparatus using the 2-ml cell (channel length 5 cm, cross section 0.30 cm^2). Patterns were photographed with a Land camera, a strip tungsten lamp serving as light source. Prior to electrophoresis the OSM solutions were dialysed at 2° for two days against two changes of the buffer used in the experiment.

RESULTS

Electrophoretic examination of OSM

Electrophoresis of OSM (1 % w/v) in phosphate buffer (pH 7.3; ionic strength 0.2) and in veronal buffer (pH 8.6; I = 0.1) revealed a single peak in both the ascending and descending limbs. The mobilities of the mucoprotein in the two buffers were -7.4 and $-8.1 \cdot 10^{-5}$ $cm^2\ sec^{-1}\ V^{-1}$ respectively. The electrophoretic patterns of OSM in veronal buffer at various time intervals are reproduced in Fig. 1. Similarly a single peak only was found on ultracentrifugal analysis. A detailed report on the behaviour of OSM in the ultracentrifuge and on the viscosity of OSM will be given in a subsequent paper.

Main components of OSM

The main components of OSM and their percentage by weight are shown in Table I.

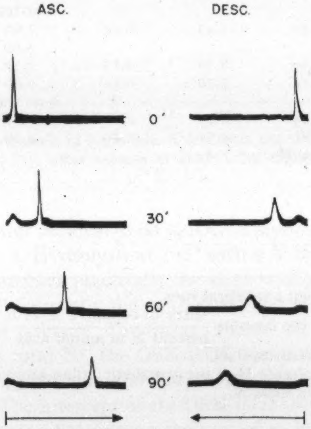

Fig. 1. Electrophoretic pattern of OSM in veronal buffer (pH 8.6; I = 0.1) at a potential gradient of 7.06 V/cm at intervals of 30 min.

TABLE I

ANALYTICAL DATA FOR OSM

	Percent of dry weight
N-acetylneuraminic acid	25.0
N-acetylgalactosamine	18.5
Carbohydrate residue	42.0*
Protein residue	58.0**
Total nitrogen	11.6
Carbohydrate nitrogen	2.3
Protein nitrogen	16.0
Sulphur	0.43

* Allowing for elimination of 1 molecule H_2O between NANA and NAGal.
** By difference.

TABLE II

AMINO ACID ANALYSIS OF OSM

Amino acid	*Amino acid nitrogen as % of total nitrogen*					
	a *20-h hydrolysis mean**	*b* *70-h hydrolysis mean**	*c* *Overall mean or extrapolated value*	*d* *Standard error*	*e* *Grams of amino acid per 100 g OSM***	*f* *Grams of amino acid residue per 100 g OSM*
Alanine	6.67	7.10	6.88	0.14	5.08	4.05
Arginine	9.21	9.14	9.18	0.20	3.31	2.97
Aspartic acid	2.81	2.72	2.76	0.04	3.04 (4.10***)	2.63 (3.55)
Cystine					1.61***	1.37
Glutamic acid	3.54	3.97	3.76	0.10	4.58 (6.17***)	4.02 (5.42)
Glycine	9.38	9.82	9.60	0.16	5.97	4.54
Histidine	0.67	0.72	0.70	0.06	0.30	0.27
Isoleucine		1.75	1.75	0.09	1.90	1.64
Leucine	2.68	2.70	2.69	0.01	2.92	2.52
Lysine	2.40	2.54	2.47	0.11	1.50	1.32
Methionine					0 or trace	
Phenylalanine		1.43	1.43	0.04	1.96	1.75
Proline					5.60***	4.72
Serine	7.76	5.39	8.70	0.13	7.57	6.27
Threonine	6.79	6.14	7.10	0.11	7.00	5.94
Tryptophan					0 or trace	
Tyrosine	0.45	0.45	0.45	0.008	0.68	0.61
Valine	4.45	4.21	4.33	0.10	4.20	3.55
"Amide"	10.49§§	14.90§§				
						48.17 (50.49)
						+ 42.00 carbohydrate
						— 1.48 water§
						88.69 (91.01)

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* Mean of three independent analytical runs.

** Calculated according to the formula $\frac{\text{entry of column c} \times 11.6.}{\text{percent N in amino acid}}$

*** Determined by independent method.

§ Allowing for loss of 1 molecule H_2O per prosthetic group-amino acid linkage.

§§ Ammonia-N in percent of total N.

Amino acid analysis of OSM

The results of the quantitative amino acid determinations are summarised in Table II. The absence of tryptophan was indicated by a negative Ehrlich reaction of the chromatogram of the alkali hydrolysate of OSM. The quantity of methionine present was too small to be measured. Though no cystine was detected in the effluent fractions from the ion-exchange columns, its presence in OSM was indicated by an appreciable sulphur content. Since the prosthetic group is free of sulphur, the sulphur content of OSM is expressed in Table II as cystine. The total dicarboxylic acids, as determined by an independent procedure (see METHODS), amounted to 10.7 % expressed as glutamic acid. This quantity was proportionated to aspartic and glutamic acids in the ratio obtained with the MOORE AND STEIN procedure; the resulting figures are given in brackets (Table II).

Amide determination in OSM

When OSM was heated in *N* HCl at 100° for various periods of time and the ammonia-N released determined quantitatively, the values shown in Fig. 2 were obtained. However, even under these mild conditions of hydrolysis the NANA component of OSM decomposed with humin formation, as was already observed previously[11]. To eliminate any possible contribution of the decomposing NANA to the ammonia formed, OSM was digested with neuraminidase prior to acid treatment.

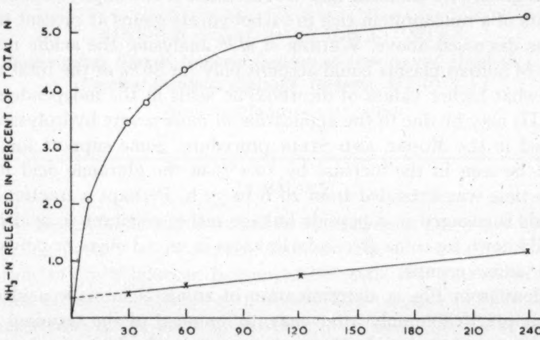

Fig. 2. Time curve of the formation of ammonia-N from 5.0 mg OSM on treatment with N HCl at 100°; O—O, before; ×—×, after release of NANA by digestion with 10,000 units RDE at 35° for 6 h.

The ammonia-N values after removal of 80 % NANA and corrected for the remaining 20 % are included in Fig. 2. Hydrolysis at 100° with 2 N HCl instead of N HCl prior to ammonia determination gave practically the same results. Linear regressions were fitted to the experimental figures by the method of least squares and estimates made of the intercept with the ordinate. The linear limb of the OSM curve extrapolates to 4.49 % N (referred to total N), the OSM-RDE curve to 0.34 % N. The slope of the latter curve is +0.2073 per hour and statistically indistinguishable from the final slope of the OSM curve. The intercept of the OSM-RDE curve, *i.e.* 0.34 % N referred to total N, is taken as amide-N following the procedure of Leach and Parkhill[12].

DISCUSSION

The protein moiety of OSM has an almost complete complement of amino acids; however, no appreciable amounts of tryptophan and methionine seem to be present. Further characteristic features of the protein are its low content of tyrosine, lysine and histidine, its relatively high content of proline and its high content of serine threonine and glycine.

Of the 11.3 (9.0) % mucoprotein not accounted for in Table II, column f, one part was degraded with the formation of ammonia during acid hydrolysis preceding the Moore and Stein fractionation and determination of the constituent amino acids. Thus, of the 10.49 % ammonia-N (referred to total N) present in the 20-h hydrolysate only 4.49 % was derived from NANA and from amide, whereas 6 % of the total N equal to 0.696 g N or 0.696 × 100/16 = 4.35 g amino acid residues/100 g OSM were deaminated in the hydrolysis procedure (6 N HCl, 100°, 20 h). Included in this figure is part at least of the 2.30 g residues of serine (0.67 g), threonine (0.26 g) and cystine (1.37 g) whose destruction is already allowed for in Table II, column f. The remaining deficit must be due to loss of amino acids by irreversible condensation with sugar constituents[13, 14] of OSM and/or in the process of column chromatography. Losses in the determination of the sugar components are allowed for in the carbohydrate figures given (see GRAHAM AND GOTTSCHALK[2]). A complete balance sheet of the constituents of a mucoprotein rich in carbohydrate seems at present not feasible for the reasons discussed above. WEIMER *et al.*[15] analysing the acidic mucoprotein (orosomucoid) of human plasma could account only for 86 % of the total weight.

The somewhat higher values of dicarboxylic acids in the independent determination (Table II) may be due to the application of more severe hydrolysis conditions than those used in the MOORE AND STEIN procedure. Some support for this interpretation may be seen in the increase by 12.1 % in the glutamic acid value when the hydrolysis time was extended from 20 h to 70 h. Perhaps a fraction of the dicarboxylic acids is engaged in a peptide linkage rather resistant to acid hydrolysis, a situation well known for some glycosidic linkages in mixed oligo- or polysaccharides containing free amino groups.

As is evident from Fig. 2, determination of amide-N in sialic acid containing mucoproteins is practicable only after enzymic removal of the terminal sialic acid residues. Once removed the amide-N may be conveniently determined according to the linear extrapolation procedure described by LEACH AND PARKHILL[12]. The amide-N of OSM is very low.

ACKNOWLEDGEMENT

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REFERENCES

[1] A. GOTTSCHALK AND S. FAZEKAS DE ST. GROTH, *J. Gen. Microbiol.*, 22 (1960) in the press.
[2] E. R. B. GRAHAM AND A. GOTTSCHALK, *Biochim. Biophys. Acta*, 38 (1960) 513.
[3] C. C. CURTAIN AND J. PYE, *Australian J. Exptl. Biol. Med. Sci.*, 33 (1955) 315.
[4] P. B. HAMILTON, *Anal. Chem.*, 30 (1958) 914.
[5] S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
[6] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
[7] D. H. SIMMONDS AND R. G. ROWLANDS, *Anal. Chem.*, in the press.
[8] R. ACHER, C. FROMAGEOT AND M. JUTISZ, *Biochim. Biophys. Acta*, 5 (1950) 81.
[9] E. J. CONWAY AND E. O'MALLEY, *J. Biol. Chem.*, 36 (1942) 655.
[10] W. ZIMMERMANN, *Mikrochemie*, 31 (1943) 15.
[11] A. GOTTSCHALK, *Yale J. Biol. and Med.*; 28 (1956) 525.
[12] S. J. LEACH AND E. M. J. PARKHILL, *Proceed. International Wool Textile Research Conference, Australia*, 1955, C-92.
[13] A. GOTTSCHALK, *Biochem. J.*, 52 (1952) 455.
[14] K. HEYNS AND M. ROLLE, *Ber.*, 92 (1959) 2439.
[15] H. E. WEIMER, J. W. MEHL AND R. J. WINZLER, *J. Biol. Chem.*, 185 (1950) 561.

ON THE BIOSYNTHESIS OF L-FUCOSE AND L-FUCOSE METABOLISM IN MAN

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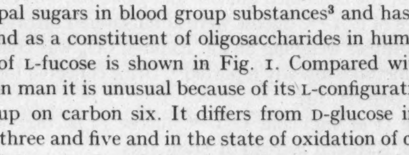
SUMMARY

Studies of the biosynthesis and metabolism of L-fucose in man have been performed. The ^{14}C labeling pattern of L-fucose in human milk subsequent to the intravenous administration of [6-^{14}C]glucose is compatible with the conversion of the intact hexose carbon chain of D-glucose to L-fucose. These results parallel those observed previously in studies of the bacterial synthesis of L-fucose. [1-^{14}C]fucose administered intravenously to a human subject was found to be rapidly and extensively converted to $^{14}CO_2$.

INTRODUCTION

The sugar, L-fucose, is known to occur in a variety of microorganisms, and in plant and animal tissues. It is a major component of the capsular polysaccharides of certain bacteria[1] and the polysaccharides of some marine algae[2]. In man, L-fucose is found as one of the principal sugars in blood group substances[3] and has been isolated both as the free sugar[4] and as a constituent of oligosaccharides in human milk[5,6].

The structure of L-fucose is shown in Fig. 1. Compared with the structure of other sugars found in man it is unusual because of its L-configuration and the absence of a hydroxyl group on carbon six. It differs from D-glucose in the configuration about carbons two, three and five and in the state of oxidation of carbon six. Previous reports from this laboratory[7] and elsewhere[8] have suggested that in *Aerobacter aerogenes*, strain A3S1, L-fucose of the capsular polysaccharide was synthesized by



L-Fucose D-Glucose

Fig. 1.

Biochim. Biophys. Acta, 42 (1960) 147–151

utilization of the intact carbon chain of D-glucose. Observations supporting this pathway of fucose biosynthesis have also been made by others studying a different microorganism[9].

An attempt has now been made to delineate the biosynthetic relationship between D-glucose and L-fucose in man by studying the formation of L-fucose in human milk. In addition, because of its presence in human milk and blood group polysaccharide, the metabolic fate of L-[^{14}C]fucose in man has also been determined.

MATERIALS AND METHODS

Four days *post partum*, after the mechanical emptying of each breast of milk, five microcuries of D-[6-^{14}C]glucose were administered intravenously to each of two lactating but non-nursing negro females on the obstetrical service of the District of Columbia General Hospital. 5 h later the breasts were again emptied and the milk obtained pooled for a total of 80 ml.

64.8 mg of L-fucose, determined by the method of DISCHE AND SHETTLES[10] were isolated from the milk by the extractive and chromatographic techniques of HEYNS, WALTER AND HEYDE[4]. Following the addition of 55.2 mg of pure L-fucose as carrier, crystalline derivatives were prepared and degraded by methods previously described[7].

Crystalline lactose obtained in the course of the fucose isolation was hydrolyzed in sulfuric acid. An aliquot of the hydrolysate was treated with glucose oxidase and gluconate isolated by the method of HEATH AND ROSEMAN[9]. Part of the gluconate was converted to the benzimidazole derivative according to MOORE AND LINK[11] and then combusted to CO_2 (see ref. 12) in order to obtain the glucose specific activity. Another aliquot of the gluconate was degraded and carbons one, two to five and six isolated as previously described[13]. An aliquot of the lactose hydrolysate was treated with periodate and, after treatment of the resulting formaldehyde with permanganate, C-6 of the combined glucose and galactose was isolated as CO_2.

Five microcuries of L-[1-^{14}C]fucose were administered intravenously to a 20 year old normal male volunteer weighing 80 kg. Expired CO_2 was collected and its radioactivity assayed by methods already reported[14]. Urine collected for 21 h after injection was counted for C^{14} (see ref. 14).

D-[6-^{14}C]glucose (1 μC/mg) and L-[1-^{14}C]fucose (3 μC/mg) were obtained from Dr. H. ISBELL of the National Bureau of Standards.

RESULTS

Biosynthesis

The distribution of ^{14}C in D-glucose and L-fucose in human milk after [6-^{14}C]-glucose administration is shown in Table I. Glucose in milk has much of the asymmetric labeling of the administered glucose. The ^{14}C pattern of L-fucose is similarly asymmetric with most of the label in the C-5,6 fragment of the methylpentose skeleton. This pattern of fucose labeling in human milk suggests that, like certain bacteria, man synthesizes L-fucose predominantly by direct conversion of D-glucose to L-fucose without rupture of the carbon chain[7-9]. The somewhat greater randomization of ^{14}C in fucose seen here has also been observed when bacteria are grown with

[6-^{14}C]glucose as the carbon source[7] and suggests the presence of a secondary pathway of fucose synthesis from smaller carbon fragments.

SCHAMBYE, WOOD AND KLIEBER[15] studying lactose synthesis in the cow have shown that the glucose in milk obtained 3 h after intravenous administration of [1-^{14}C] glucose has about 90 % of the radioactivity in carbon one. These investigators, as well as others[16], suggest that glucose in milk is in equilibrium with blood glucose. Recent studies in man[17] have demonstrated that there is little randomization of ^{14}C in blood glucose for at least 3 h after intravenous injection of [6-^{14}C] glucose. It would thus appear that in man as well as the cow[15] and goat[16] glucose in milk is derived principally from blood glucose.

TABLE I

^{14}C DISTRIBUTION IN HEXOSES IN HUMAN MILK

Sugar	*Fragment*	*Specific activity counts/min/mM*	*Percent of radioactivity*
D-glucose	C-1,2,3,4,5,6	2780	
	C-1	150	4*
	C-2,3,4,5	300	10
	C-6	2700	86
L-fucose	C-1,2,3,4,5,6	2180	
	C-1	131	6
	C-2,3,4	459	22
	C-5,6	1510	72
Lactose hydrolysate	C-6	2700	

* These percentages are calculated on the basis of the sum of the fragments.

Studies of milk lactose of the cow[15], goat[16] and rabbit[18] after administration of [^{14}C]glucose have revealed an almost identical specific activity of galactose and glucose. If the assumption is made that these hexoses in human milk have similar specific activities, then the identical specific activity of C-6 in glucose with that of C-6 isolated from the equimolar mixture of glucose and galactose in the lactose hydrolysate implies that the pattern of labeling of the galactose in human milk is similar to that of the glucose. Such a conclusion is consistent with that of others[15] who have studied lactose formation in the cow and have observed that, after intravenous injection of a specifically labeled glucose, the galactose and glucose in milk had a similar pattern of isotope distribution.

Metabolism

The pattern of $^{14}CO_2$ excretion after L-[1-^{14}C]fucose administration is shown in Fig. 2. Integration of the area beneath the curve reveals that 39 % of the injected radioactivity was excreted as $^{14}CO_2$ in 6 h. 30 % of the ^{14}C given appeared in the urine within 6 h, virtually no ^{14}C being detectable in the urine after this time (Table II). If the administered ^{14}C be corrected for the urinary loss, the total $^{14}CO_2$ excreted in 6 h represents 56 % of the activity retained in the body.

Urine obtained in the first hour after the fucose injection was chromatographed on paper (methyl-ethyl ketone–acetic acid–water (6:1:1); R_F of L-fucose = 0.17) and the area corresponding to L-fucose was eluted with water and assayed for ^{14}C. 83 % of the radioactivity applied to the paper was recovered in this area (usual recovery of ^{14}C sugars so treated is about 93 % (see ref. 19)). It thus appears that most of the urinary ^{14}C represents unaltered L-fucose. Fucose may be normally found in human urine[20].

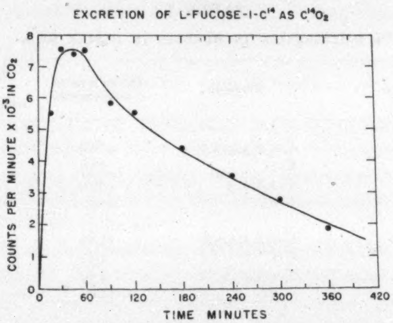


Fig. 2. The excretion of L-[1-^{14}C]fucose as ^{14}CO$_2$ after the injection of 5 μC into a normal human.

TABLE II

URINARY EXCRETION OF ^{14}C

Hour	*Percent of dose*
1	25
2	28
3	29
4	30
5	30
6	30
7–21	31

DISCUSSION

L-Fucose comprises from 14 to 19 % of various blood group polysaccharides[21]. Its presence is significant from an immunologic aspect, since it appears to be an important factor for the specificity of both H[22] and Le^a activity[23]. The destruction of blood group activity of A, B and H substance by mild acid hydrolysis is associated with liberation of fucose from the molecule.

Human milk contains about 128 mg percent L-fucose[6]. Except for the sheep, other animal milks tested thus far do not contain fucose[4]. Although fucose is of importance in the immunologic reactions involving blood group substances, the importance of L-fucose in human milk has not been ascertained. The quantity present in human milk, however, does not suggest a caloric importance of L-fucose.

The similarity in the pattern of labeling of L-fucose and D-glucose in human milk after the injection of [6-^{14}C]glucose is compatible with the interpretation that L-fucose is synthesized from D-glucose without rupture of the carbon chain. A recent report by GINSBURG AND KIRKMAN[24] has demonstrated in *Aerobacter aerogenes*, strain

A3S1, the presence of guanosine diphosphate fucose and the formation of the latter nucleotide from guanosine diphosphate mannose[25]. It thus appears that glucose is converted to guanosine diphosphate mannose with inversion of the configuration about carbon two. The latter nucleotide, by a series of reactions involving inversion of C-3 and C-5 plus reduction of C-6, is converted to guanosine diphosphate fucose. Guanosine diphosphate fucose has been isolated from sheep milk[26]. The results of the isotopic study here reported suggest the possibility of a similar mechanism of fucose biosynthesis in the human.

The fact that the fucose specific activity is less than the glucose specific activity, although the labeling pattern is similar, deserves some comment. Such a finding could be due to synthesis of fucose in part from unlabeled carbon fragments. An alternate explanation is that the fucose in milk has a slower turnover than the glucose moiety of lactose.

The present study reveals that carbon one of fucose is readily metabolized by man to CO_2. The $^{14}CO_2$ excretion curve presented here differs from that obtained from the oxidation of ^{14}C pentoses labeled in carbon one[19], as well as from [1-^{14}C]-glucose[17]. The extent and rapidity of the oxidation exceeds that of [1-^{14}C]glucose, as well as that of the pentoses, excepting ribose.

The oxidation of L-fucose appears to be extensive also in the intact rabbit and in rabbit liver and kidney slices[27]. Experiments employing *E. coli*[28] indicate that the initial steps in fucose metabolism involve the formation of the keto sugar phosphate, fuculose-1-phosphate, with subsequent conversion of the latter sugar to a product capable of being split by aldolase, presumably to dihydroxyacetone phosphate and lactaldehyde[28].

REFERENCES

[1] J. F. Wilkinson, W. F. Dudman and G. O. Aspinall, *Biochem. J.*, 59 (1955) 446.
[2] E. G. V. Percival and A. G. Ross, *J. Chem. Soc.*, (1950) 717.
[3] A. Bendich, E. A. Kabat and A. E. Bezer, *J. Am. Chem. Soc.*, 69 (1947) 2163.
[4] K. Heyns, W. Walter and W. Heyde, *Z. physiol. Chem., Hoppe-Seyler's*, 304 (1956) 279.
[5] R. Kuhn, *Angew. Chem.*, 64 (1952) 493.
[6] F. H. Malpress and F. E. Hythen, *Biochem. J.*, 68 (1958) 708.
[7] S. Segal and Y. J. Topper, *Biochim. Biophys. Acta*, 25 (1957) 419.
[8] J. F. Wilkinson, *Nature*, 180 (1957) 995.
[9] E. C. Heath and S. Roseman, *J. Biol. Chem.*, 230 (1958) 511.
[10] Z. Dische and L. B. Shettles, *J. Biol. Chem.*, 175 (1948) 595.
[11] S. Moore and K. P. Link, *J. Biol. Chem.*, 133 (1940) 293.
[12] D. D. van Slyke, J. Plazin and J. R. Weisiger, *J. Biol. Chem.*, 191 (1951) 299
[13] F. Eisenberg Jr., *J. Am. Chem. Soc.*, 76 (1954) 5152.
[14] S. Segal and J. B. Foley, *J. Clin. Invest.*, 37 (1958) 719.
[15] P. Schambye, H. G. Wood and M. Klieber, *J. Biol. Chem.*, 226 (1957) 1011.
[16] D. K. Reiss and J. M. Bary, *Biochem. J.*, 55 (1953) 783.
[17] S. Segal and A. Blair, in preparation.
[18] T. H. French, G. Popjak and F. H. Malpress, *Nature*, 169 (1952) 71.
[19] S. Segal and J. B. Foley, *J. Clin. Invest.*, 38 (1959) 407.
[20] A. A. White and W. C. Hess, *Arch. Biochem. Biophys.*, 64 (1956) 57.
[21] W. T. J. Morgan and W. M. Watkins, *Brit. Med. Bull.*, 15 (1959) 109.
[22] W. M. Watkins and W. T. J. Morgan, *Nature*, 169 (1952) 825.
[23] W. M. Watkins and W. T. J. Morgan, *Nature*, 180 (1957) 1038.
[24] V. Ginsburg and H. N. Kirkman, *J. Am. Chem. Soc.*, 80 (1958) 3481.
[25] V. Ginsburg, *J. Am. Chem. Soc.*, 80 (1958) 4426.
[26] R. Denamur, G. Fauconneau and G. Guntz, *Compt. Rend.*, 246 (1958) 2820.
[27] Y. J. Topper and S. Segal, unpublished data.
[28] P. C. Hunag and O. N. Miller, *J. Biol. Chem.*, 231 (1958) 201.

Short Communications

Carbon dioxide fixation in the synthesis of aspartic acid by a strain of *Staphylococcus aureus*

During investigations on the pathway of glucose metabolism in cells of *Staphylococcus aureus* (strain Duncan), which will be described in detail in a further paper, the distribution of carbon from [^{14}C]glucose among the compounds present in hot-water extracts of the organism was determined. Glucose, generally labelled with ^{14}C (final specific activity, 1 μC/μmole), was added to cultures growing exponentially in peptone–glucose medium[1]; after 1 h hot-water extracts of the cells were prepared and the compounds present were separated by two-dimensional paper chromatography using the solvent systems phenol–water (500 g/112 ml) followed by *n*-butanol–acetic acid–water (7:2:5, v/v/v). The radioactive compounds were located by radioautography and identified initially by reference to the position of known markers. Among the many compounds which became labelled, radioactivity was found in a compound whose position corresponded exactly with that of aspartic acid; no radioactivity was detectable in the positions of glutamic acid or of any of the intermediates of the tricarboxylic acid cycle. These results, together with others which show that production of $^{14}CO_2$ from [6-^{14}C]glucose by this organism is very small in comparison with that from [1-^{14}C]glucose and glucose generally labelled with ^{14}C, suggest that the tricarboxylic acid or glyoxalate cycles do not operate in this organism to any significant extent.

The most likely alternative route for incorporation of carbon from glucose into aspartic acid appeared to be that *via* the formation of oxaloacetate from pyruvate, involving fixation of CO_2; such a mechanism has been shown to occur in a number of species of *Lactobacilli*[2–4] and in the minute *Streptococci*[5]. It was found that exponentially-growing cells of *S. aureus* (strain Duncan) incorporate radioactivity from

TABLE I

INCORPORATION OF RADIOACTIVITY FROM $^{14}CO_2$ BY GROWING ORGANISMS

3-ml cultures of *S. aureus* (strain Duncan) were grown in PG medium[1] in Warburg manometer vessels. When the bacterial density reached about 0.2 mg/ml, $^{14}CO_2$ (initial specific activity, 7,500 counts/min/μmole) was liberated from $Ba^{14}CO_3$ in the side arm by addition of 1 *N* H_2SO_4 from the hollow stopper. After 2 h the organisms were harvested and fractionated[1]; the (protein + cell wall) fraction was hydrolysed (6 *N* HCl, 105°, 15 h) and this hydrolysate and the hot-water extract were separated by paper chromatography[1]. The values in the table are averages from 2 cultures.

Fraction	*Radioactivity (counts/min/ml culture)*	*% of total radioactivity in aspartic acid*
Hot-water extract (100°, 10 min)	2600	93.2
Soluble in hot trichloroacetic acid (90°, 6 min)	44	—
Protein + cell wall mucopeptide	4250	95.5

$^{14}CO_2$, most of the radioactivity appearing in the hot-water-extractable and protein fractions. Over 90 % of the radioactivity in both of these fractions was found to be present in aspartic acid, identified by its chromatographic behaviour and also by its sensitivity to the specific aspartic decarboxylase of *Nocardia globerula*[6] (Table I). In the hot-water-extractable fraction, the only other component which became radioactive corresponded in position with oxaloacetic acid; in the protein fraction a weakly radioactive spot appeared in the area of the chromatogram occupied by glycine and serine. When the [^{14}C]aspartic acid, isolated from the protein fraction by preparative paper chromatography, was degraded using aspartic decarboxylase[6], 92 % of the radioactivity was released as $^{14}CO_2$, showing that it was present originally in the γ-carboxyl position of L-aspartic acid.

We investigated if this incorporation of $^{14}CO_2$ represented net synthesis of aspartic acid, or an exchange reaction, *via* oxaloacetate, with aspartic acid present in the growth medium. For this purpose, a larger quantity of [γ-^{14}C]aspartic acid was prepared by incorporation of $^{14}CO_2$ into growing cells, and purified by paper chromatography. The production of $^{14}CO_2$ from this [γ-^{14}C]aspartic acid was compared with the incorporation of $^{14}CO_2$, using suspensions of non-growing organisms to prevent removal of aspartic acid into cell protein. Table II shows that under these conditions

TABLE II

REVERSIBILITY OF INCORPORATION OF $^{14}CO_2$ INTO ASPARTIC ACID

Growing organisms were harvested and incubated in Warburg flasks at a concentration of about 5 mg/ml in defined growth medium[7] containing chloramphenicol (200 μg/ml). In one flask (A) the aspartic acid included [γ-^{14}C]aspartic acid prepared by $^{14}CO_2$ incorporation. In the other flask (B), $^{14}CO_2$ was liberated from $Ba^{14}CO_3$. After 2 h 0.5 ml N H_2SO_4 was tipped into both flasks and CO_2-free 7 N KOH was introduced through the hollow stopper into the side arm of flask A. After a further hour the absorbed CO_2 in flask A was precipitated as $BaCO_3$, washed and counted. The initial specific activity of the CO_2 in flask B was estimated using a second identical flask.

Reaction		*Specific activity of precursor (counts/min/μmole)*	*Total counts/min in product*
[γ-^{14}C]aspartic acid → $^{14}CO_2$	(A)	10,150	770
$^{14}CO_2$ → [γ-^{14}C]aspartic acid	(B)	1,510*	508

* Initial specific activity; dilution with respiratory CO_2 would reduce this figure finally to at least 1 % of this value. The value for the amount of CO_2 fixed is therefore a minimal one.

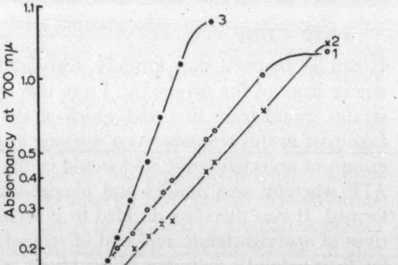


Fig. 1. Growth of *S. aureus* (strain Duncan) in defined medium[7] with aspartic acid (200 μg/ml) (cultures 1 and 3) or omitting aspartic acid (cultures 2 and 4). Cultures 1 and 2 were aerated slowly with air; a rapid stream of CO_2-free air (passed successively through 7 N KOH, 1 N $Ba(OH)_2$ and distilled water) was passed through cultures 3 and 4.

the amount of $^{14}CO_2$ produced from [γ-^{14}C]aspartic acid is very small compared with the incorporation of $^{14}CO_2$; the incorporation therefore involves a net fixation of CO_2.

This conclusion is further supported by observations on the interrelations between CO_2 and aspartic acid in the growth of this organism (Fig. 1). When a defined medium[7] was used, with or without aeration, there was no difference in the growth rate when aspartic acid was omitted from the medium. When precautions were taken to exclude CO_2 from the culture medium, and a stream of CO_2-free air was passed through the culture, the growth rate and the final cell density in the absence of aspartic acid were virtually zero, indicating that fixation of CO_2 is the main pathway of synthesis of aspartic acid in this organism under these conditions.

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[1] J. T. PARK AND R. HANCOCK, *J. Gen. Microbiol.*, 22 (1960) 249.
[2] H. A. LARDY, *J. Biol. Chem.*, 179 (1949) 721.
[3] V. H. LYNCH AND M. CALVIN, *J. Bacteriol.*, 63 (1952) 525.
[4] J. C. MACDONALD, *Can. J. Microbiol.*, 4 (1958) 335.
[5] W. R. MARTIN AND C. F. NIVEN, *J. Bacteriol.*, 79 (1960) 295.
[6] L. V. CRAWFORD, *Biochem. J.*, 68 (1958) 221.
[7] R. HANCOCK, *Biochim. Biophys. Acta*, 28 (1958) 402.

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The catalytic effect of glutamic acid derivatives in urea synthesis

From the experiments of JONES *et al.*[1] it seems most probable that the first step in the synthesis of urea in mammalian liver leads to the formation of carbamyl phosphate. This step is followed by a reaction of carbamyl phosphate with ornithine to form citrulline. The earlier work, emanating from the laboratory of COHEN, has demonstrated convincingly that the first step requires the presence of a cofactor with catalytic properties which was identified as acetylglutamic acid[2], though related derivatives of this compound also exerted catalytic activities[3]. The enzymes responsible for this reaction[4] (see below) were recently extracted from frog liver[5,6]:

$$2\,ATP + NH_3 + CO_2 \xrightarrow{\text{acetylglutamic acid}} 2\,ADP + \text{carbamyl phosphate} + H_3PO\text{[illegible]} \quad (1)$$

It can be assumed that initially acetylglutamic acid is involved in a reaction with one or more of the substrates. From this the question arises which of the three substrates would react first and which of the molecular groups of the cofactor would take part in this reaction. As a working hypothesis it was thought that the carboxyl groups of acetylglutamic acid would in the first place react with either NH_3 or with ATP whereby acid amides and phosphoric acid anhydrides respectively would be formed. It was therefore decided to synthesize various amide and anhydride derivatives of acetylglutamic acid and of related compounds and to test such compounds for their catalytic activities in the above reaction.

The references given below refer to known methods used for the synthesis of

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Abbreviations: ATP, ADP, adenosine tri- and diphosphate.

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the following compounds: N-acetylglutamic acid[7,8]; N-carbamylglutamic acid[9]; N-carbamylglutamine[10]; N-carbobenzoxyglutamic acid[11]; L-isoglutamine and N-carbobenzoxy-L-isoglutamine[12]; 3-acetylhydantoin-5-propionic acid lactam[13].

Three compounds were synthesized as follows:

N-acetylglutamine. A suspension of L-glutamine in pyridine was treated with acetyl chloride under cooling and shaking. To the resulting mixture ether was added, the precipitate formed was taken up in weak alkaline solution and the pyridine was removed by distillation. The oily residue was taken up in ethanol, ether was added until an oil separated which was discarded; more ether was added to the remaining solution until an amorphous precipitate could finally be separated; a solution of the precipitate gave no ninhydrin reaction. M.p., 185–190°.

N-acetylisoglutamine. This was prepared from isoglutamine by treatment with acetic anhydride. The acidified mixture was evaporated to dryness, the residue was taken up in ethanol and reprecipitated with ethyl acetate. Both the precipitate and the supernatant contained acetylisoglutamine in about equal quantities as ascertained by paper chromatography. The presence of traces of N_1N'-diacetylisoglutamine in the preparation was revealed by preparing its hydroxamic acid derivative[14] and by applying the chlorination test[15,16].

α- and γ-N-acetylglutamyl phosphate. Acetylglutamic acid was treated with hot acetic anhydride until completely dissolved. After removal of the latter and of the acetic acid by distillation, the residue was stirred in the cold with KH_2PO_4 and pyridine until the substance was completely dissolved. By adding a solution of 4 *N* LiOH the lithium salt of the phosphorylated compound was formed, inorganic phosphate was removed by precipitation with ethanol in the cold and the final product was obtained by further treatment with ethanol. By estimating quantitatively the hydroxamic acids[14] prepared from the components of the preparation and the heat-labile phosphate[17], it was found that the final product contained a mixture of the α- and γ-phosphates as well as some unchanged acetylglutamic acid which could not be separated.

Hydantoin-5-propionic acid was a gift of Dr. H. WAELSCH, Columbia University, New York.

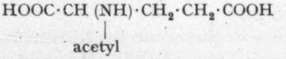
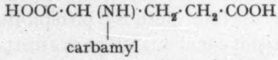
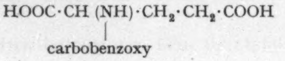
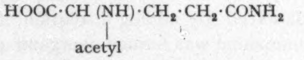
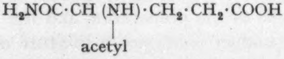
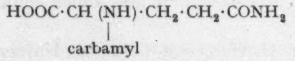
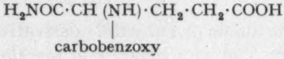
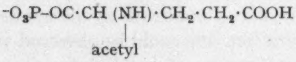
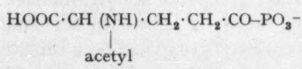
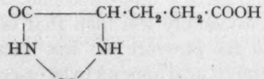
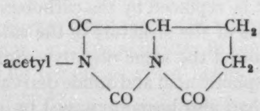
From Table I it will be seen that none of the amide or anhydride derivatives of glutamic acid was able to replace acetylglutamic acid as a catalyst in reaction (1) and they cannot therefore be regarded as intermediary compounds in the synthesis of carbamyl phosphate. Only a very slight catalytic activity could be observed with compounds 6 and 8 in which one of the carboxyl groups of acetylglutamate was substituted with ammonia. Carbamylglutamate, which is known to act similarly to acetylglutamate[3], became inactive when its γ-carboxyl group had been substituted with ammonia (compound 9). The activity of the mixture of 1- and 5-phosphorylated acetylglutamate (compounds 11a and 11b) was considerably less than that of the phosphate-free catalyst and was obviously due to the presence of "free" acetylglutamate (see preparation). The acyl group of the catalyst acid appears to be essential, since compounds 3, 5 and 7 were inactive, nor can it be replaced by the carbobenzoxy group (compounds 4 and 10). More drastic alterations of the structure of the catalyst through ring formation (compounds 12 and 13) showed the same negative result.

The possibility of the primary formation of phosphoric acid and amide derivatives during the catalytic action of acetylglutamic acid had also been envisaged by other

TABLE I

THE CATALYTIC EFFECT OF GLUTAMIC ACID DERIVATIVES ON THE SYNTHESIS OF CITRULLINE

Composition of incubation mixture (quantities in µmoles): ATP, 4; 3-phosphoglycerate, 30; L-ornithine, 20; NH_4Cl, 30; $NaHCO_3$, 100; $MgCl_2$, 20; phosphate buffer, pH 7.4, 20; glutamic acid derivative, 10. Liver enzyme, 1 ml; muscle enzyme (for regeneration of ATP), 0.4 ml; total volume, 2 ml. Incubation time, 40 min at 37°. After incubation the mixture was deproteinised by adding 5 ml 0.5 *M* $HClO_4$ and the citrulline estimated according to ARCHIBALD[23]. The citrulline synthesis was considered a measure of the catalytic activities of the test substances. The liver enzyme was prepared by extracting 1 g ox liver acetone powder with 10 ml water. 0.3 % rabbit muscle extract was prepared according to RATNER AND PAPPAS[24].

No. of compound			*Citrulline synthesis*
			%
1	 $\mathrm{HOOC\cdot CH}\underset{\substack{\vert \\ \text{acetyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot COOH}$	N-acetyl-L-glutamic acid	100
2	 $\mathrm{HOOC\cdot CH}\underset{\substack{\vert \\ \text{carbamyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot COOH}$	N-carbamyl-L-glutamic acid	35
3	$\mathrm{HOOC\cdot CH\,(NH_2)\cdot CH_2\cdot CH_2\cdot COOH}$	L-glutamic acid	0
4	 $\mathrm{HOOC\cdot CH}\underset{\substack{\vert \\ \text{carbobenzoxy}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot COOH}$	N-carbobenzoxy-L-glutamic acid	1
5	$\mathrm{HOOC\cdot CH\,(NH_2)\cdot CH_2\cdot CH_2\cdot CONH_2}$	L-glutamine	0
6	 $\mathrm{HOOC\cdot CH}\underset{\substack{\vert \\ \text{acetyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot CONH_2}$	N-acetyl-L-glutamine	7
7	$\mathrm{H_2NOC\cdot CH\,(NH_2)\cdot CH_2\cdot CH_2\cdot COOH}$	L-isoglutamine	0
8	 $\mathrm{H_2NOC\cdot CH}\underset{\substack{\vert \\ \text{acetyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot COOH}$	N-acetyl-L-isoglutamine	1
9	 $\mathrm{HOOC\cdot CH}\underset{\substack{\vert \\ \text{carbamyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot CONH_2}$	N-carbamyl-L-glutamine	
10	 $\mathrm{H_2NOC\cdot CH}\underset{\substack{\vert \\ \text{carbobenzoxy}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot COOH}$	N-carbobenzoxy-L-isoglutamine	1
11a	 $\mathrm{^{-}O_3P\text{–}OC\cdot CH}\underset{\substack{\vert \\ \text{acetyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot COOH}$	mixture of: 1- and 5-N-acetyl-L-glutamyl phosphate	62
b	 $\mathrm{HOOC\cdot CH}\underset{\substack{\vert \\ \text{acetyl}}}{\mathrm{(NH)}}\mathrm{\cdot CH_2\cdot CH_2\cdot CO\text{–}PO_3^{-}}$		
12		hydantoin-5-propionic acid	1
13		3-acetylhydantoin-5-propionic acid lactam	0

workers[18,19]. More recently, METZENBERG *et al.*[5,20] denied this possibility and further concluded that ammonia did not take part in the first phase of the catalysis, a view also shared by JONES[21].

Thus, free carboxyl groups are prerequisite for the catalytic action of acetylglutamic acid; further, since the carbon-bonded hydrogen atoms do not take part in the catalysis[22], the reactivity of acylglutamic acid seems to be associated with its substituted amino group.

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[1] M. E. JONES, L. SPECTOR AND F. LIPMANN, *J. Am. Chem. Soc.*, 77 (1955) 819.
[2] L. M. HALL, R. L. METZENBERG AND P. P. COHEN, *J. Biol. Chem.*, 230 (1958) 1013.
[3] S. GRISOLIA AND P. P. COHEN, *J. Biol. Chem.*, 204 (1953) 753.
[4] R. L. METZENBERG, L. M. HALL, M. MARSHALL AND P. P. COHEN, *J. Biol. Chem.*, 229 (1957) 1019.
[5] R. L. METZENBERG, M. MARSHALL AND P. P. COHEN, *J. Biol. Chem.*, 233 (1958) 1560.
[6] M. MARSHALL, R. L. METZENBERG AND P. P. COHEN, *J. Biol. Chem.*, 233 (1958) 102.
[7] P. KARRER, K. ESCHER AND A. WIDMER, *Helv. Chim. Acta*, 9 (1926) 301.
[8] B. H. NICOLET, *J. Am. Chem. Soc.*, 52 (1930) 1192.
[9] H. A. KREBS, L. V. EGGLETON AND V. A. KNIVETT, *Biochem. J.*, 59 (1955) 185.
[10] P. P. COHEN AND S. GRISOLIA, *J. Biol. Chem.*, 182 (1950) 747.
[11] S. GOLDSCHMIDT AND C. JUTZ, *Ber.*, 86 (1953) 1120.
[12] T. WIELAND AND H. WEIDENMÜLLER, *Ann. Chem., Liebigs*, 597 (1955) 111.
[13] D. L. SZABO AND J. V. KARABINOS, *J. Am. Chem. Soc.*, 66 (1944) 650.
[14] F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
[15] H. N. RYDEN AND P. W. G. SMITH, *Nature*, 169 (1952) 922.
[16] T. WIELAND, *Angew. Chem.*, 66 (1954) 787.
[17] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[18] S. GRISOLIA, in W. D. MCELROY AND AND B. GLASS, *Phosphorus Metabolism*, Vol. I Baltimore, 1951, p. 619.
[19] S. GRISOLIA AND J. C. TOWNE, *Biochim. Biophys. Acta*, 25 (1957) 224.
[20] R. L. METZENBERG, M. MARSHALL, P. P. COHEN AND W. G. MILLER, *J. Biol. Chem.*, 234 (1959) 1534.
[21] M. E. JONES, *Federation Proc.*, 18 (1959) 255.
[22] S. GRISOLIA, R. H. BURRIS AND P. P. COHEN, *J. Biol. Chem.*, 210 (1954) 761.
[23] R. M. ARCHIBALD, *J. Biol. Chem.*, 156 (1944) 121.
[24] S. RATNER AND A. PAPPAS, *J. Biol. Chem.*, 179 (1949) 1183.

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Hexose monophosphate pathway in pituitary tissue

Few studies have been made on glucose catabolism in the pituitary. Rat pituitary is characterized by a high endogenous respiration, which is unaffected by the presence of glucose or succinate in the medium[1,2]. The concentration of the enzymes of the tricarboxylic acid cycle is significantly smaller than in liver[2].

An active hexose monophosphate pathway has been demonstrated in the adrenals[3], in the testis[4], and in the thyroid[5], and the hypothesis has been suggested that this might be a common property of endocrine tissues. The present study presents evidence for an active hexose monophosphate pathway in beef pituitary.

The experimental methods were the same as those previously reported. The recorded isotopic values (Table I) have been corrected to the initial specific activity of [1-^{14}C]glucose.

As shown in Table I, there is a preferential oxidation of the C-1 in contrast to the C-6 of glucose by beef pituitary. The ratio of $^{14}CO_2$ evolved from [1-^{14}C]glucose to that from [6-^{14}C]glucose is approx. 3 after 30, 60, and 90 min incubation. If glucose were metabolized *via* the glycolytic and citric acid pathway, a ratio of 1 would have been observed as in muscle. There is thus good evidence for the existence of an active hexose monophosphate pathway in pituitary tissue.

TABLE I

OXIDATION OF [1-^{14}C]GLUCOSE AND [6-^{14}C]GLUCOSE BY BEEF PITUITARY

Each flask contained 4 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), 52.8 μmoles glucose and approx. 90 mg of tissue slices (dry wt.), equilibrated with 95 % O_2, 5 % CO_2. Specific activity of glucose was 0.023 C/mole glucose.

Tissue	*Incubation time (min)*	*Specific activity* of CO_2 formed from*		*Ratio C-1/C-6*	*Number of experiments*
		[1-^{14}C]glucose	*[6-^{14}C]glucose*		
Beef pituitary	30	0.494 ± 0.240**	0.155 ± 0.121**	3.19	8
Beef pituitary	60	1.922 ± 0.565**	0.840 ± 0.290**	2.29	8
Beef pituitary	90	3.709 ± 0.591**	1.219 ± 0.482**	3.04	6
Rat diaphragm	90	19.7	23.7	0.83	1

* Specific activity in counts/min/mg $BaCO_3$/mg tissue (dry wt.).
** Standard deviation.

The steadiness of the C-1/C-6 ratio permits the assumption that this ratio at zero time was close to 3. Assuming that the triose phosphates are freely interconvertible, that there is no randomization of C-1 to C-4, C-5, or C-6, and that the rate of formation of CO_2 is higher from C-1 than from C-2 and C-3 in the hexose monophosphate pathway, it is possible to evaluate grossly[6] that 25 % of the CO_2 produced from glucose comes via this pathway.

It is of interest to point out that both thyroid and pituitary tissues have a low concentration of the enzymes of the tricarboxylic acid cycle[2,7], and an active hexose monophosphate pathway[5].

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[1] S. ROBERTS AND M. R. KELLER, *Arch. Biochem. Biophys.*, 44 (1953) 9.
[2] J. B. MELCHIOR AND D. M. HILKER, *J. Biol. Chem.*, 212 (1955) 187.
[3] T. L. KELLY, E. D. NIELSON, R. B. JOHNSON AND C. S. VESTLING, *J. Biol. Chem.*, 212 (1955) 545.
[4] B. BLOOM, *Proc. Soc. Exptl. Biol. Med.*, 88 (1955) 317.
[5] J. E. DUMONT, *Biochim. Biophys. Acta*, 40 (1960) 354.
[6] S. KORKES, *Ann. Rev. Biochem.*, 25 (1956) 685'
[7] J. E. DUMONT, in preparation.

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Stereospecificity of the sugar-phosphate isomerase reactions; a uniformity

It has been shown for all the phosphoaldose isomerases studied thus far that the interconversion of aldose and ketose in deuterated or tritiated water results in the incorporation of one atom of isotope in the C-2 and C-1 positions respectively[1–3]. Furthermore, it has been shown that the position labeled on C-1 of fructose 6-phosphate formed from glucose 6-phosphate with phosphoglucose isomerase is not the same as that labeled in the reaction of mannose 6-phosphate with phosphomannose isomerase[1].

The absolute configuration of the monotritiated dihydroxyacetone phosphate formed with triose phosphate isomerase in tritiated water has been established by conversion of that ketose to glycolic acid through HIO_4 oxidation and further oxidation to glyoxylic acid by the stereospecific reaction with glycolic acid oxidase[4]. In the present report, a similar approach has been used to determine the stereospecificity of labeling of fructose 6-phosphate and ribulose 5-phosphate formed by reaction with phosphoglucose isomerase and phosphopentose isomerase respectively. It is the purpose of this work to determine whether there is any uniformity underlying the stereospecificity of the different isomerase reactions.

D-ribose 5-phosphate was treated with spinach phosphopentose isomerase[5] in tritiated water until equilibrium had been established. The mixture was treated with HIO_4 and the glycolate isolated by ion-exchange chromatography and converted to glyoxylate enzymically[4]. D-[1-^{3}H]glucose was phosphorylated with yeast hexokinase and the isolated glucose 6-phosphate was treated with phosphoglucose isomerase until equilibrium amounts of fructose 6-phosphate had been formed. This mixture was then treated as above for the eventual isolation of glyoxylate derived from the first two carbons of the fructose 6-phosphate.

The results of this pair of experiments (Table I) show that in the phosphoglucose isomerase reaction the proton from water is not found in the final glyoxalate (that is, tritium is retained), and that in the phosphopentose isomerase reaction the tritium

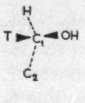
TABLE I

ANALYSIS OF GLYCOLIC AND GLYOXYLIC ACIDS DERIVED FROM EQUILIBRIUM MIXTURES OF PHOSPHOGLUCOSE AND PHOSPHORIBOSE ISOMERASE REACTION

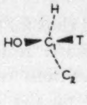
In the first experiment D-ribose 5-phosphate (30 μmoles) was incubated 15 min in 0.3 ml ^3H_2O (156,000 counts/min/μatom hydrogen) with phosphopentose isomerase (an amount of enzyme to convert 10 μmoles substrate/min), 0.06 *M* triethanolamine-acetate buffer, pH 7.5, and 3.5 m*M* cysteine. In the second experiment D-[1-^{3}H]glucose 6-phosphate (6.3 μmoles and containing 140,000 counts/min/μmole) was incubated with phosphoglucose isomerase (an amount of enzyme to convert 3 μmoles of substrate/min) and 0.05 *M* triethanolamine-acetate, pH 7.5 in a final volume of 1.5 ml for 15 min at 37°. Both incubations were treated with HIO_4 and the remaining procedure for isolation and counting is as previously described[5].

Expt.	*Specific activity of glycolic acid (counts/min/μmole)*	*Specific activity of glyoxylic acid (counts/min/μmole)*	*% of tritium specifically located*
1. phosphoribose isomerase	178,000	7,525	96
2. phosphoglucose isomerase	90,000	107,000	108

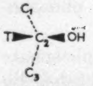
derived from the water is lost in the oxidase step. From these data, the previous report on phosphotriose isomerase, and the observation that phosphomannose isomerase and phosphoglucose isomerase act on different C-1 positions of fructose 6-phosphate it may be concluded that dihydroxyacetone phosphate, D-ribulose 5-phosphate, and D-fructose 6-phosphate derived respectively from reaction of triosephosphate isomerase, phosphopentose isomerase and phosphoglucose isomerase in tritiated water have the configuration around C-1 shown in Fig. 1a, whereas fructose-6-phosphate derived from the phosphomannose isomerase reaction in tritiated water is as shown in Fig. 1b. On the other hand, it is clear from the structure of the aldose-phosphates corresponding to the above sequence that the first three, D-glyceraldehyde 3-phosphate, D-ribose 5-phosphate, and D-glucose 6-phosphate formed in tritiated water have the configuration at C-2 shown in Fig. 2a and that D-mannose 6-phosphate is as shown in Fig. 2b. Thus a correlation seems to exist among the four enzymes for which data is available between the configuration of the aldose and that of the derived, tritiated ketose.



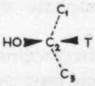
1a



1b



2a



2b

It has been proposed[1] that the mechanism of the isomerase reactions might involve the intermediate formation of a 1,2 enediol bound to the enzyme. If this is assumed to be correct then the present results may be generalized in the following terms: (a) If a cis-enediol is formed, proton attack on C-1 or C-2 must occur from the same side of the plane of the enediol structure. (b) If a trans-enediol is formed, proton attack on C-1 or C-2 must occur from opposite sides of the plane of the enediol structure. (c) A cis-enediol intermediate cannot be attacked by protons from opposite sides nor can a trans-intermediate be attacked at each carbon from the same side.

In the paper of Topper[1] it was suggested that the difference in stereospecificity of phosphoglucose and phosphomannose isomerases required that the enediols formed be cis in one case and trans in the other. However, it is possible that only (a) or (b) holds in both cases if one provides that opposite faces of the plane of the same enediol are bound in the complex with each enzyme.

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[1] Y. J. Topper, *J. Biol. Chem.*, 225 (1957) 419.
[2] S. V. Rieder and I. A. Rose, *J. Biol. Chem.*, 234 (1959) 1007.
[3] W. A. Wood and M. W. McDonough, *Federation Proc.*, 18 (1959) 354.
[4] I. A. Rose, *J. Am. Chem. Soc.*, 80 (1958) 5835.
[5] J. Hurwitz, A. Weissbach, B. L. Horecker and P. Z. Smyrniotis, *J. Biol. Chem.*, 218 (1956) 769.

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Distribution of nucleic acids among subcellular fractions of *Chlorella*[*]

In a previous study[1] it was observed that the base compositions of RNA and DNA fractions obtained from *Chlorella* cells by the SCHMIDT-THANNHAUSER[2] method change significantly in the course of synchronous growth. To pursue this observation further, cells of *Chlorella ellipsoidea* were disrupted and fractionated into three subcellular components, and the nucleic acids were isolated from these three fractions. The distributions and some characteristics of the nucleic acids are reported here.

Chlorella cells were disrupted with a French pressure cell (Amercian Instrument Co., Inc., Silver Spring, Md.) at 0°–4° in a medium containing 70 mM KCl, 2 mM Ca^{++}, and 5 mM Mg^{++}. Over 95 % of the cells were disrupted by being put through the apparatus twice. The ruptured cell material was centrifuged at 0° for a short period to eliminate the remaining whole cells and cell debris. Then it was centrifuged successively at 20,000 × g for 10 min and at 105,000 × g for 90 min to give pellets R-I and R-II, respectively. The first pellet (R-I) was dark green and consisted mainly of broken chloroplasts. The second pellet (R-II) was pale green and the final supernatant fluid was clear and amber. An example of chemical analyses on the three fractions is shown in Table I. RNA is distributed in all three fractions, but DNA is found almost exclusively in R-I and S. R-II consists mainly of ribonucleoprotein particles. The ribonucleoprotein particles obtained in a sucrose medium (0.20 M sucrose, 2 mM Ca^{++}) and which can be degraded with RNase had sedimentation coefficients ($s_{20,w}$) of about 40, 55 and 75 S. Preliminary assays showed that the relative amount of RNA in the three fractions and of DNA in the two fractions that contained it varied in the course of synchronous growth.

TABLE I

CHEMICAL ANALYSES ON THE THREE CELL FRACTIONS OF *Chlorella ellipsoidea*

Cell materials: 3.6 g in dry weight.

Material	*Amount of material in cell fraction (mg)*			*Whole homogenate*
	R-I	*R-II*	*S*	
Chlorophyll[*]	50	0.03	0.00	55
RNA[**]	22	27	8.4	61
DNA[**, ***]	3.1	0.36	3.5	7.5
Protein[§]	510	85	200	820

[*] Methanol extractions.
[**] SCHMIDT-THANNHAUSER method[2].
[***] DISCHE diphenylamine reaction[2].
[§] Biuret reaction.

The same distribution of DNA into two fractions as shown in Table I was also found in homogenates produced by alumina grinding[3] and by the Hughes press[4], or when other disrupting media were used (*e.g.*, 0.1–0.5 M sucrose or 0.02–0.15 M salts in combination with 0, 10^{-4}, and $2 \cdot 10^{-3}$ M Ca^{++} and 0, 10^{-3}, and $5 \cdot 10^{-3}$ M Mg^{++}).

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

[*] A part of this communication was presented to the Federation of American Societies for Experimental Biology (American Society for Biological Chemistry), Atlantic City, N.J., April 13–17, 1959.

A fractionation of the disrupted cell material with a sucrose gradient (from 10 to 60 %) at 1700 rev./min for 30 min[5] gave the same result and no DNA was detected in cell components heavier than the broken chloroplasts (R-I). The broken chloroplasts purified by repeated differential centrifugation contained DNA as well as RNA, and they were stained a diffuse reddish purple with the Feulgen reagent, while the whole cell was stained by the dye in one or several locations.

The nucleic acids were isolated by detergent extraction (a modification of the KAY AND DOUNCE method[6,7]) with Duponol C (E. I. DU Pont De Nemours and Co., Inc., Wilmington, Del.) (see ref. 4). About 60 % of the total nucleic acids was usually obtained as ethanol-precipitable, nondialysable material. The nucleic acids thus obtained were hydrolyzed with alkali, and the DNA was separated from the ribonucleotides of the RNA by precipitation with acid and solubilization with alkali three times. The results are shown in Table II. The RNA in the supernatant contains more cytosine and less adenine than the RNA of the particulate fractions, and the DNA in R-I (DNA-I) contains more thymine and adenine than the DNA in the supernatant (DNA-II).

TABLE II

BASE COMPOSITIONS OF THE NUCLEIC ACIDS ISOLATED FROM THE THREE CELL FRACTIONS OF *Chlorella ellipsoidea*[1]

Analyses from several samples obtained by using different methods, each run in duplicate: RNA (5 samples) by ion-exchange and paper chromatography of ribonucleotides[8,9]; DNA (4 samples) by paper chromatography of bases after digestion with 72 % $HClO_4$ (3 samples)[9] and ion-exchange chromatography of deoxyribonucleotides[8] after DNAse and venom digestion (1 sample)[10].

Nucleic acid	*Base composition*	*Range of mole per cent in*		
		R-I	*R-II*	*S*
RNA	Uracil	23–24	23–24	21–24
	Adenine	24–27	23–27	18–24
	Cytosine	23–25	22–24	26–30
	Guanine	24–30	27–30	25–32
DNA	Thymine	22–28		19–21
	Adenine	24–28		21–23
	Cytosine	24–28		27–30
	Guanine	20–28		28–30

The phosphorus metabolism of the two DNA fractions was examined with ^{32}P tracer. An inoculum for the synchronous culture[1] prepared in unlabeled medium was grown at 16° with $^{32}PO_4$ present under a saturating light intensity, and the cells were harvested at 17 h when they were increasing in mass but were not synthesizing DNA (Expt. 1). Another inoculum that had grown in a ^{32}P medium for three generations was grown synchronously in an unlabeled medium, and the cells were harvested at 52 h when they had just begun to synthesize DNA[1] (Expt. 2). In both cases the two fractions containing DNA were isolated and digested with DNAase and snake venom phosphodiesterase (kindly supplied by Dr. L. ASTRACHAN of this Laboratory), and the specific activity of the separated (individual) deoxyribonucleotides was measured. The results are shown in Table III. Here it can be seen that DNA-I turns over faster than DNA-II in terms of phosphorus.

TABLE III

LABELING AND DELABELING OF DNA-I AND DNA-II WITH ^{32}P

In specific activity: counts/min/μmole P.

Expt.	*DNA-I*	*DNA-II*
1*	3700 (1610–5300)**	1200 (560–1870)
2***	390 (100–700)	1800 (1100–2440)

* A nonlabeled inoculum grown synchronously in a ^{32}P medium. The specific activities of the whole cells and of the culture medium at the harvest were 21,000 and 130,000 counts/min/μmole P, respectively.

** Figures in parentheses are the maximum and minimum specific activities for the four constituent nucleotides, the average of which appears above them.

*** A labeled inoculum grown synchronously in the unlabeled medium. The specific activities of the whole cells at the start of the culture and at harvest were 1200 and 280 counts/min/μmole P, respectively. At harvest, the DNA content per cell was 1.3 times as great as that at the start.

From all these findings, we conclude that DNA exists in two physically separable subcellular components in *Chlorella* cells and that the two DNA's differ from each other in metabolic activity as well as in base composition. It is also possible that DNA-I exists in or on the chloroplasts, whereas DNA-II is in the cytoplasmic portion, probably in a nucleus-like body in the cytoplasm of *Chlorella* cells. (Shortly after we had completed the work reported here, STOCKING AND GIFFORD presented a paper in which they demonstrated the uptake of labeled thymidine by the chloroplasts of the alga *Spirogyra*[10]).

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[1] T. IWAMURA AND J. MYERS, *Arch. Biochem. Biophys.*, 84 (1959) 267.
[2] E. VOLKIN AND W. E. COHN, in D. GLICK, *Methods of Biochemical Analysis*, Vol. I, Interscience Publishers, Inc., New York, 1954, p. 287.
[3] H. MCILWAIN, *J. Gen. Microbiol.*, 2 (1948) 288.
[4] D. E. HUGHES, *Brit. J. Exptl. Pathol.*, 32 (1951) 97.
[5] J. F. ALBRIGHT AND N. G. ANDERSON, *Exptl. Cell Research*, 15 (1958) 271.
[6] E. R. M. KAY AND A. L. DOUNCE, *J. Am. Chem. Soc.*, 75 (1953) 4041.
[7] E. R. M. KAY, N. S. SIMMONS AND A. L. DOUNCE, *J. Am. Chem. Soc.*, 74 (1952) 1724.
[8] W. E. COHN, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I, Academic Press Inc., New York, 1955, Chap. 6.
[9] R. MARKHAM, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. IV, Springer Verlag, Berlin, 1955, p. 246.
[10] G. SCHMIDT, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I, Academic Press Inc., New York, 1955, Chap. 15.
[11] C. R. STOCKING AND C. M. GIFFORD, JR., *Biochem. Biophys. Research Commun.*, 1 (1959) 159.

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Preliminary Notes

Solubilization of aromatic hydroxylase system of liver microsomes and requirement of lipid-like factor

In attempts to elucidate the enzymic mechanisms involved in the hydroxylation of foreign aromatic compounds by liver microsomes[1], it was desirable to obtain the hydroxylase system in a soluble form. We have, therefore, tested a variety of methods and succeeded in solubilizing the aniline-hydroxylating activity of liver microsomes with the aid of heat-treated venom of *Trimeresurus flavoviridis*. Since this venom, like those of other snakes, is known to contain heat-stable phospholipase A[2], it is likely that this enzyme is the principal agent responsible for the solubilization. Digestion of microsomes with the untreated venom, on the other hand, resulted in a complete inactivation of the enzyme system probably owing to the action of powerful proteases present in the venom[3]. The other methods tested including treatments with pancreatic lipase, bile salts, several synthetic detergents, digitonin, *n*-butanol, and sonic oscillation all either caused inactivation or brought about insufficient solubilization of the hydroxylase system.

The microsomes used were prepared from rabbit liver and suspended in 1.15 % KCl according to MITOMA *et al.*[4]. They were usually kept at 4° for about 20 h prior to digestion, since it was noted that microsomes thus aged are somewhat more readily solubilized than fresh preparations. The heat-treated venom was prepared by heating a crude venom solution (1 % in 0.05 *M* Tris, pH 7.4) for 8 min in a boiling-water bath and then removing the resulting precipitate by centrifugation. The aniline-hydroxylating activity was measured as in the previous report[1] using glucose 6-phosphate, glucose 6-phosphate dehydrogenase and TPN as a TPNH-generating system.

A mixture containing 11 ml of microsomal suspension (equivalent to 20 g liver), 4 ml 0.5 *M* Tris, pH 9.0, and 1 ml of the heat-treated venom was incubated at 4° for 16 to 18 h. The digest was then adjusted to pH 7.5 (no measurable solubilization takes place at this pH), diluted to 60 ml, and subjected to centrifugation at 105,000 × *g* for 60 min. More than 90 % of the hydroxylase activity and approximately 60 % of microsomal protein were recovered in the supernatant thus obtained. Spectrophotometric investigations further showed that both cytochrome b_5 and DPNH–cytochrome b_5 reductase had also been solubilized during the digestion.

The 105,000 × *g* supernatant could be dialysed against 0.002 *M* Tris buffer (pH 7.5) for more than 3 h without appreciable loss of activity. It was further possible to lyophilize the dialysed preparation to give a reddish powder, the activity of which was quite stable when stored in the cold in a desiccator.

In a previous communication[1] it was shown that a lipid-like component indispensable for the hydroxylase activity can be extracted from microsomes with acetone–methanol–ether and that the activity of microsomes thus treated can be

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

restored by the addition of the component extracted in the solvent mixture. It was thus of interest to see whether the solubilized preparation behaves in the same way. Such experiments seemed especially desirable in view of our experiences that the extraction experiments with intact or acetone-dried microsomes were sometimes (notably in winter) not quite reproducible for reasons still to be elucidated.

Solvent extraction of the solubilized system was therefore conducted as follows: 100 mg of the lyophilized powder obtained above was suspended in 50 ml of cold acetone–methanol–ether (7:1:2, v/v/v) and homogenized for 15 min at — 10 to — 15°. The homogenate was filtered and the residue reextracted with another 50-ml portion of the cold solvent. The residue after second extraction was dried over conc. H_2SO_4 in a vacuum desiccator, whereas the combined extracts were mixed with a small amount of water and concentrated *in vacuo* to give about 1 ml of an aqueous lipid emulsion.

TABLE I

LIPID-LIKE FACTOR REQUIREMENT IN SOLUBILIZED ANILINE HYDROXYLASE SYSTEM

The reaction mixture contained 0.1 *M* Tris-acetate buffer (pH 8.5), 4 µmoles aniline, 8 µmoles nicotinamide, 0.12 µmole TPN, 2.5 µmoles $MgCl_2$, 7.5 µmoles glucose 6-phosphate, 0.64 unit glucose 6-phosphate dehydrogenase, 0.35 ml solubilized enzyme system, and indicated amount of lipid emulsion (obtained as described in the text). The total volume was 1.0 ml. The mixture was aerobically shaken for 60 min at 37°. Hydroxylation was followed by measuring *p*-aminophenol formation as described previously[1].

Solubilized enzyme sustem		*Lipid emulsion (ml)*	*p-Aminophenol formed (mµmole)*	*Spec. activity (mµmole p-amino-phenol/mg protein)*
Treatment	*Protein (mg)*			
Solvent-extracted	2.17	0	7.3	3.4
Solvent-extracted	2.17	0.05	27.7	12.8
Solvent-extracted	2.17	0.10	39.4	18.2
Untreated	2.26	0	40.3	17.9

It was thus found, as can be seen from Table I, that the aniline-hydroxylating activity of the lyophilized powder was greatly diminished by the extraction, but could be fully restored by the addition of the lipid emulsion.

These experiments, so far invariably reproducible, again indicate an important role played by a lipid-like factor in the microsomal hydroxylation. Furthermore, they are of special significance in that these results can be obtained even with those microsomes which fail to give an active lipid-like factor on direct extraction and thus provide a more convenient means for the study of the factor.

We wish to thank Drs. S. MITSUHASHI and H. MAENO for a generous gift of the snake venom.

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[1] Y. IMAI AND R. SATO, *Biochim. Biophys. Acta*, 36 (1959) 571.
[2] A. OHSAKA, *J. Biochem. (Tokyo)*, 45 (1958) 259.
[3] H. MAENO, S. MITSUHASHI, Y. SAWAI AND T. OKONOGI, *Japan. J. Microbiol.*, 3 (1959) 131.
[4] C. MITOMA, H. S. POSNER, H. C. REITZ AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 61 (1956) 431.

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Effects of prolactin *in vitro* on the metabolism of glucose by rat mammary gland

It has long been known that prolactin is concerned in the initiation of lactation. Moreover, a direct local action of prolactin on the secretory activity of mammary alveoli has been demonstrated by LYONS[1]. BALMAIN AND FOLLEY[2] investigated the effect *in vitro* of prolactin on the metabolism of slices of rat mammary gland at different stages of lactation. Although the respiration of tissue taken at 1–5 days lactation was sensitive to the addition of prolactin to the medium they failed to find any metabolic changes, in the respiratory quotient or in the incorporation of labelled acetate into lipids, in slices of tissue from pregnant ratos.

In view of the intraduct-injection results of LYONS[1] it might be expected that prolactin would have an effect *in vitro* on the metabolism of mammary tissue from pregnant rats and for this reason measurements have been made of the action of this hormone on the conversion of glucose carbons I and 6 to CO_2 and lipid and the relative participation of the Embden–Meyerhof and pentose phosphate pathways in the conversion of glucose to lipid by mammary-gland slices from pregnant and lactating rats. The results of these experiments are shown in Fig. 1.

The addition of prolactin to the medium caused a significant increase in the formation of $^{14}CO_2$ from both [1-^{14}C]glucose and [6-^{14}C]glucose and also increased the incorporation of glucose carbon into lipid by mammary-gland slices from pregnant

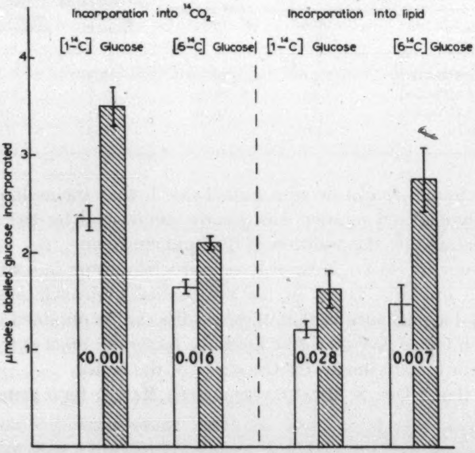

Fig. 1. Effect of prolactin on the conversion of specifically labelled glucoses to $^{14}CO_2$ and lipid by mammary-gland slices from pregnant rats. 500 mg mammary-gland slices were incubated for 1.5 h at 37° in 4.5 ml Krebs–Ringer bicarbonate solution with 100 μmoles glucose containing 0.2 μC ^{14}C in the form of [1-^{14}C]glucose or [6-^{14}C]glucose. Gas phase, O_2–CO_2 (95:5). Open columns represent values for control slices, hatched columns those for slices incubated with 3 mg prolactin/flask. (Armour "Panlitar" lot S10209 15 units/mg). The vertical lines represent twice the standard error of the mean and the figures across adjacent columns are the values for Fisher's P calculated using the method of corresponding pairs (number of pairs = 7). Method of incubation and isotopic procedure were as described previously[6] except that the total lipid was extracted by the method of BLIGH AND DYER[7] and was counted using the "infinitely thick" layer technique.

rats. The amount of ^{14}C-labelled lipid derived from added glucose arising from the Embden-Meyerhof pathway has been calculated by the method described by ABRAHAM *et al*[3]. While 85 ± 6 % arose from glycolysis in control experiments this value fell to 58 ± 6 % in experiments in which prolactin was added to the tissue slices (Fisher's $P = 0.023$). By difference it is apparent that the contribution of the non-glycolytic route is more than doubled in the presence of prolactin.

In contrast with the above results prolactin had no consistent effect *in vitro* on the metabolism of specifically labelled glucoses by mammary-gland slices from lactating rats (11th–18th day).

WINEGRAD *et al.*[4] have recently shown that prolactin *in vitro*, in concentrations similar to those used in the present study, increased both the oxidation of glucose carbon to $^{14}CO_2$ and the incorporation of glucose carbon into fatty acids by adipose tissue from normal-fed rats. The present results are also in accord with those of ABRAHAM *et al.*[5] who have investigated the oxidation of glucose and acetate and lipogenesis from these substrates by mammary glands of hypophysectomised rats in which lactation was hormonally induced. These authors found that administration of prolactin and Δ^1-hydrocortisone acetate together brought about pronounced changes in the pattern of glucose metabolism, a greater proportion of the glucose-derived fatty acids arising from the pentose phosphate pathway.

It is of interest that the effect of prolactin disappears with the onset of lactation while the action of insulin is considerably enhanced[6], a point of contrast which suggests that these two hormones have different mechanisms of action although the general pattern, the parallel increase in the oxidation of glucose and synthesis of lipid, is apparent in both cases.

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[1] W. R. LYONS, *Proc. Soc. Exptl. Biol. Med.*, 51 (1942) 308.
[2] J. H. BALMAIN AND S. J. FOLLEY, *Arch. Biochem. Biophys.*, 39 (1952) 188.
[3] S. ABRAHAM, P. F. HIRSCH AND I. L. CHAIKOFF, *J. Biol. Chem.*, 211 (1954) 31.
[4] A. I. WINEGRAD, W. N. SHAW, F. D. W. LUKENS AND W. C. STADIE, *J. Biol. Chem.*, 234 (1959) 3111.
[5] S. ABRAHAM, P. CADY AND I. L. CHAIKOFF, *Endocrinology*, 66 (1960) 280.
[6] P. MCLEAN, *Biochim. Biophys. Acta*, 37 (1960) 296.
[7] E. G. BLIGH AND W. J. DYER, *Can. J. Biochem. Physiol.*, 37, (1959) 911.

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The mode of action of actinomycin D

Actinomycin D is one of a series of polypeptide antibiotics, the first of which was reported by WAKSMAN AND WOODRUFF in 1940[1]. It has powerful bacteriostatic effects on many micro-organisms, particularly Gram-positive bacteria.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; d.ATP, deoxyadenosine triphosphate; d.GTP, deoxyguanosine triphosphate; d.TPP, thymidine triphosphate; d.CTP, deoxycytosine triphosphate; Tris, tris (hydroxymethyl)aminomethane.

At concentrations of 0.25–0.75 μg/ml it completely inhibits growth of *Staphylococcus aureus* strain Duncan. Respiration and glycolysis of washed suspensions of this organism are unaffected by concentrations of up to 100 μg/ml, but in a synthetic medium[2], containing amino acids, purines, pyrimidines and glucose, the net synthesis of protein, inducible β-galactosidase, and nucleic acids is inhibited at growth-inhibitory concentrations. The extent of the inhibition obtained with a given concentration of the antibiotic is dependent on the density of the cell suspension used.

Addition of actinomycin D to an exponentially-growing culture of *Staph. aureus* produces an immediate inhibition of the synthesis of RNA, followed rapidly by inhibition of protein synthesis. Inhibition of DNA synthesis occurs after a lag period of some 10–15 min, but the degree of inhibition obtained for a given concentration of actinomycin D is less than that occurring for RNA or protein synthesis. Actinomycin D also inhibits the incorporation of [^{14}C]labelled amino acids, purines or pyrimidines into the hot-trichloroacetic acid-insoluble and -soluble fractions, the effects correlating with those on net protein and nucleic acid synthesis. Incorporation of [^{14}C]glutamic acid into the cell wall material of *Staph. aureus*, isolated as described by PARK AND HANCOCK[3], is however unaffected by actinomycin D at concentrations of up to 20 μg/ml. The inhibitory action of actinomycin D on the incorporation of ^{14}C-labelled substances into the "protein" and "nucleic acid" fractions is antagonised by the addition of a preparation of staphylococcal nucleic acid to the incubation mixture. Fractionation and purification of the nucleic acid indicates that the antagonism is entirely due to the DNA fraction. DNA preparations from calf thymus, wheat germ, herring roe, *Brucella* and *Bacillus megaterium* also have this antagonistic effect.

The addition of DNA to a solution of actinomycin D at pH 7.0 reduces the absorption of the latter, and shifts the position of the absorption maximum towards the red end of the spectrum. The complex formed appears to be freely dissociable. Equilibrium dialysis[4] indicates that the absorption maximum of the actinomycin-DNA complex is about 465 mμ, and that, in a solution containing 136 μg/ml actinomycin D and 600 μg/ml DNA at pH 7.0, 84 % of the actinomycin is in the complexed form. Actinomycinic acid, the dicarboxylic acid derived from actinomycin D, is without antibacterial action, and the addition of DNA has no effect on its absorption spectrum. Bacterial RNA (prepared as described in ref. 5, 6), heat-denatured DNA, and heparin have no effect on the biological activity or on the absorption spectrum of actinomycin D.

Investigation of the possible metabolic consequences of the reaction between actinomycin D and DNA indicates that (i) the antibiotic has no significant inhibitory effect on the polynucleotide phosphorylase of *Staphylococcus aureus* in the system described by GRUNBERG-MANAGO, ORTIZ AND OCHOA[7]; (ii) as shown in Fig. 1, actinomycin D inhibits the incorporation of radioactivity into the $HClO_4$-insoluble fraction when [^{32}P]d.ATP is incubated with a crude preparation of the DNA "polymerase" enzyme isolated from *Escherichia coli* in an assay system similar to that described by LEHMAN, BESSMAN, SIMMS AND KORNBERG[8], (iii) actinomycin D inhibits the transformation of *Haemophilus influenzae* Graciae from streptomycin sensitivity to streptomycin resistance when it is added to high concentrations of transforming DNA from a resistant strain. As shown in Fig. 2, at lower DNA concentrations actinomycin has no effect on the percentage of cells transformed compared with the control. The lack of inhibition at lower DNA concentrations might be due to dissociation of the actinomycin–DNA complex in very dilute solutions.

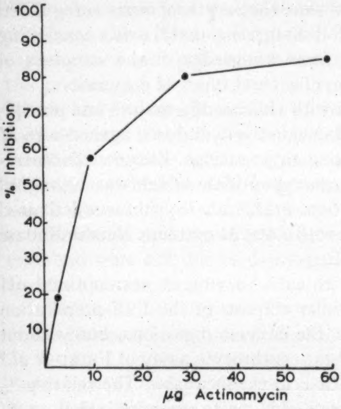

Fig. 1. Inhibition of conversion of [^{32}P]d.ATP into an acid-insoluble product when crude preparation of DNA "polymerase" is incubated at 37° for 60 min. The incubation mixture (0.3 ml) contained 10 µl enzyme preparation, 2 µmoles MgCl$_2$, 20 µmoles glycine buffer, pH 9.2, 0.3 µmole cysteine, 10 µg thymus DNA, about 3.0 µmoles [^{32}P]d.ATP, 6.6 µmoles d.GTP, 2.9 µmoles d.TPP, 4.3 µmoles d.CTP, 5.5 µmoles Tris buffer, pH 7.5 containing 11 µg thymus DNA.

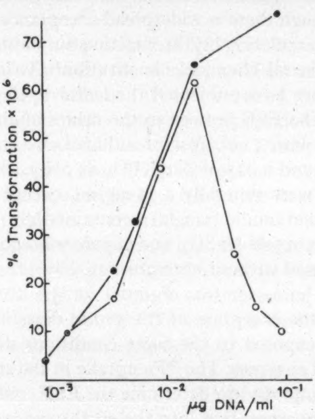

Fig. 2. The effect of actinomycin D on the transformation of *Haemophilus influenzae* from streptomycin sensitivity to streptomycin resistance. ●, in the absence of actinomycin; ○, in the presence of actinomycin at a ratio actinomycin/DNA of 50:1.

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[1] S. A. WAKSMAN AND H. B. WOODRUFF, *Proc. Soc. Exptl. Biol. Med.*, 45 (1940) 609.
[2] E. F. GALE AND J. P. FOLKES, *Biochem. J.*, 55 (1953) 721.
[3] J. T. PARK AND R. HANCOCK, *J. Gen. Microbiol.*, 22 (1960) 249.
[4] I. M. KLOTZ, F. M. WALKER AND R. B. PIVAN, *J. Am. Chem. Soc.*, 68 (1946) 1486.
[5] A. S. JONES, *Biochim. Biophys. Acta,* 10 (1953) 607.
[6] S. K. DUTTA, A. S. JONES AND M. STACEY, *Biochim. Biophys. Acta,* 10 (1953) 613.
[7] M. GRUNBERG-MANAGO, P. J. ORTIZ AND S. OCHOA, *Biochim. Biophys. Acta,* 20 (1959) 269.
[8] I. R. LEHMAN, M. J. BESSMAN, E. S. SIMMS AND A. KORNBERG, *J. Biol. Chem.*, 233 (1958) 163.

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Enzymic actions on the human urinary erythropoietic-stimulating factor

Although there is widespread acceptance of the view that erythropoiesis is regulated by a circulating erythropoietic-stimulating factor, disagreement still exists concerning its general chemical constitution[1]. To obtain some knowledge of the structure of ESF we have subjected the factor to the action of several types of enzymes.

The ESF present in the urines of children with thalassemia major[2] was precipitated with 4 vol. absolute ethanol at —10° and exhaustively dialyzed against 0.16 M NaCl and 0.025 M Na_2HPO_4 at pH 7.4 in Visking 20/32 casings. Enzyme concentrations were generally 0.75 mg/ml, except for carboxypeptidase which was 1.5 mg/ml and neuraminidase which contained 1.3 μg, or 6000 units/ml[*]. Trypsin was activated with 0.015 M $MgSO_4$, and papain was activated with 0.005 M cysteine. Neuraminidase was used without activator.

The factor was digested for 5 h at 37° with each enzyme at near optimal pH with the exception of the peptic digestion. Similar aliquots of the ESF preparation were exposed to the same conditions used for the enzyme digestions, but without added enzymes. The ^{59}Fe uptake in the starved-rat-erythrocyte assay of FRIED *et al.*[3] was employed to determine the ESF concentrations in the incubates. The relative % ^{59}Fe incorporation into the erythrocytes of groups of 3–5 rats receiving 2 daily 2-ml injections of the incubation mixtures is shown in Table I.

It is apparent that the urinary ESF is more stable in alkaline than in acid solution. For this reason, a suboptimal pH was selected for the peptic digestion. In general,

TABLE I

EFFECT OF DIGESTION UPON ACTIVITY OF HUMAN URINARY ESF

pH	*Enzyme added*	*Relative % ^{59}Fe uptake*
8.2	None	100.0
7.4	None	87.6
6.0	None	68.2
4.7	None	40.9
4.7	Pepsin	12.7[*]
7.4	Trypsin	6.6[*]
6.0	Papain	0.0[*]
7.4	Carboxypeptidase	108.0
8.2	Aminopeptidase	33.1[*]
7.4	Ribonuclease	97.0
6.0	Lysozyme	103.1
4.7	Hyaluronidase	27.7
6.0	Neuraminidase	22.1[*]

[*] Significantly lower in activity ($P < 0.05$) compared to the pH control.

Abbreviation: ESF, erythropoietic-stimulating factor.

[*] Crystalline pepsin, trypsin, ribonuclease and carboxypeptidase-DFP were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Lysozyme and hyaluronidase were secured from the Mann Research Laboratories, New York and the leucine aminopeptidase was supplied by the Bios Laboratories, Inc., New York. Neuraminidase was kindly prepared and supplied by Dr. G. L. ADA, University of Melbourne, Australia.

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the proteolytic enzymes, pepsin, trypsin, papain and aminopeptidase, but not carboxypeptidase, produced significant inactivation. Among the non-proteolytic enzymes studied, hyaluronidase and neuraminidase diminished activity, whereas ribonuclease and lysozyme were ineffective.

From these data, it is inferred that the active portions of ESF consist of both carbohydrate and polypeptide moieties. Susceptibility to aminopeptidase suggests the presence of an unblocked polypeptide approach to the active region from an N-terminal position. Resistance of the C-terminal position to carboxypeptidase could have occurred if this end were blocked by carbohydrate or by a slowly lysed peptide bond. Inactivation with neuraminidase indicates that the terminal carbohydrate derivative of human ESF is neuraminic acid in agreement with LOWY *et al.*[4] who noted that rabbit plasma ESF is also susceptible to neuraminidase.

Evidence that the effects of these enzymes were exerted directly upon ESF *in vitro* and were not due to depression of erythropoiesis within the recipient rats has also been obtained and will be included with complete details in a forthcoming paper.

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[1] A. S. GORDON, *Physiol. Rev.*, 39 (1959) 1.
[2] J. WINKERT, A. S. GORDON, P. T. MEDICI, S. J. PILIERO, A. L. LUHBY AND M. TANNENBAUM, *Proc. Soc. Exptl. Biol.-Med.*, 97 (1958) 191.
[3] W. FRIED, L. F. PLZAK, L. O. JACOBSON AND E. GOLDWASSER, *Proc. Soc. Exptl. Biol. Med.*, 92 (1956) 203.
[4] P. H. LOWY, G. KEIGHLEY AND H. BORSOOK, *Nature*, 185 (1960) 102.

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Negative-staining electron microscopy of proteins at pH values below their isoelectric points. Its application to hemocyanin

The negative-staining technique of BRENNER AND HORNE[1] is a very important method for studying the structure of protein molecules with the electron microscope. In this method contrast is obtained by the use of potassium phosphotungstate at pH 7.2. Because of the negative charge of the phosphotungstate ions, this method is applicable only at pH values above the isoelectric point, where the protein molecules also carry negative charges and thus do not take up the contrasting ion.

We are studying the structure of the molecules of *Helix pomatia* hemocyanins at different pH values and wished to extend this method below the isoelectric point. Uranyl acetate appeared to give the best result as a contrasting agent. It is clearly seen from Fig. 1 that uranyl ions do not interact with the protein. Uranyl acetate seems to be amorphous and gives a high contrast. The projections of the molecules appear as circles with a diameter of 300 Å and squares with a side of 300 Å. Their substructure is clearly visible. It is to be expected that this method will be generally applicable to proteins and other macromolecules with a positive charge below pH 5.

A discussion of these results and those of other experiments on hemocyanins will be published shortly.

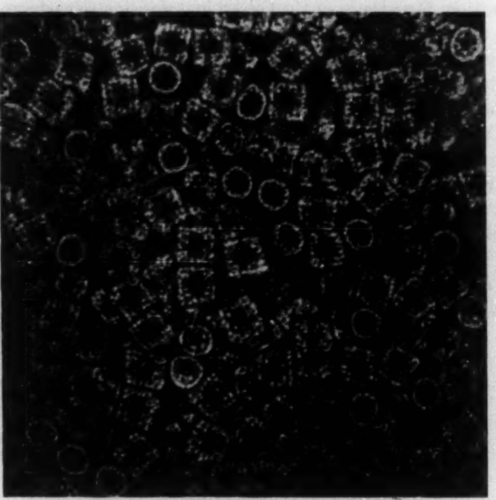

Fig. 1. Hemocyanin molecules (× 210,000). One droplet of the dialysed and centrifuged blood of *Helix pomatia* was mixed with 2 ml of a solution containing 0.5 % uranyl acetate and 0.1 % ammonium acetate, which was brought to a pH 4.5 by the addition of dilute acetic acid The mixture was immediately sprayed on a carbon-coated specimen grid and studied in the new Philips EM-200 electron microscope.

We wish to thank Miss V. E. M. Schuiten for her skilful technical assistance, the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) for a grant to one of us (v.B.), and The Laboratory of Microscopic Research of N.V. Philips at Eindhoven for kindly giving us the opportunity to use their EM-200 electron microscope.

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[1] S. Brenner and R. W. Horne, *Biochim. Biophys. Acta*, 34 (1959) 103.

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The amino acid sequence around the serine phosphate in phosphoglucomutase

PGM was prepared[1] and labelled with ^{32}P by two methods using the principle of the exchange of phosphate with the substrates[2]. Peptides from [^{32}P]PGM were obtained by partial acid hydrolysis (5.7 *N* HCl for 30 min in a boiling-water bath), subjected to ionophoresis (40 V/cm for 2 h in pyridine–acetate buffer, pH 3.5) and radioautographed. A sample of denatured PGM was subjected to the same labelling and subsequent treatment and used as control. From a tryptic digest of [^{32}P]PGM, a radioactive band was isolated by ionophoresis at pH 6.5. It was also hydrolysed and run in parallel with the hydrolysates described above (Fig. 1). For comparison, the pattern obtained from [^{32}P]DIP-chymotrypsin which had been subjected to exactly the same procedure is shown also. From this pattern and from one obtained by ionophoresis at pH 6.5 it can be concluded that there is no similarity[3] between the sequence around the serine phosphate in PGM and the reactive serine in chymotrypsin. Both Ser^{32}P·Gly and Asp·Ser^{32}P dipeptides are absent in the partial hydrolysates of [^{32}P]PGM.

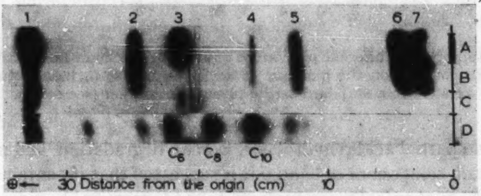

Fig. 1. Radioautograph of ionograms (Whatman No. 52 paper, pyridine–acetate buffer pH 3.5, 40 V/cm, 2 h) of partial acid hydrolysates of ^{32}P derivatives; A, [^{32}P]PGM; B, main radioactive band from a tryptic digest of [^{32}P]PGM (isolated by ionophoresis at pH 6.5); C, denatured PGM subjected to the same labelling and subsequent treatment as A; D, [^{32}P]DIP-chymotrypsin; the Asp·SerP (C6), Asp·SerP·Gly (C8), and SerP·Gly (C10) bands are indicated[5].

Isotopic techniques similar to those used in the studies of the sequence in proteolytic enzymes[4,5] have been used to characterise the radioactive bands present in the hydrolysate. In Fig. 1, band 1 is phosphate and band 3 runs parallel with a glucose 6-phosphate marker. Band 2 parallels a serine phosphate marker in ionophoresis at pH 2.1, 3.5, and 6.5. Finally bands 4, 5, 6 and 7 all yield band 2 after further acid hydrolysis. Bands 6 and 7 are a mixture of at least 2 and 3 components respectively, which were separated and studied further.

When the main component, present in band 7, was rehydrolysed (45 min in 5.7 *N* HCl at 100°) it gave three bands in the positions of the unchanged peptide, serine phosphate and phosphate, respectively. This suggests that it is a dipeptide. The main radioactive product after Edman degradation is phosphate, which is characteristic of peptides with N-terminal serine phosphate[4]. From mobility studies at different pH values it appears to contain two ionizable groups, each with a pK

Abbreviations: PGM, phosphoglucomutase; SerP, serine phosphate; DIP, diisopropyl phosphate residue.

between 5 and 7 (Fig. 2). One is the second acidic group of the phosphate residue and the other corresponds most probably to a basic amino acid residue. After purification of this peptide by ionophoresis at pH 3.5, 6.5 and 2.1, the radioactive spot was found to give a positive Pauly reaction. From these and other considerations to be published, the structure of the peptide is believed to be SerP·His.

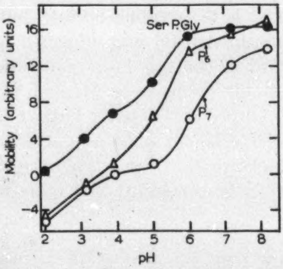

Fig. 2. Relative mobilities at different pH values of some of the main radioactive components of bands 6 and 7. For comparison the mobility of SerP·Gly is also included. Mobilities expressed relative to a standard serine phosphate marker (see ref. 5).

Finally, from partial acid rehydrolysis, Edman degradation, and mobility studies (Fig. 2) of the main component of band 6 it is suggested that it contains the tripeptide built up by addition of an acidic amino acid to the C-terminal histidine residue of the above dipeptide. Two neutral amino acids appear to precede serine phosphate in the peptide chain. The exact nature of these amino acids is under investigation.

The location of one histidine residue next to the active serine is of interest since it has been suggested that histidine is part of the active centre[6], and there are only three such residues present in the molecule[7] (mol. wt., 74,000). It has also been described as part of the active centre in several esterases and reaction mechanisms have been proposed based on the presence of a histidine residue sterically near to the reactive serine[8].

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[1] V. NAJJAR, *J. Biol. Chem.*, 175 (1948) 281.
[2] V. JAGANATHAN AND J. M. LUCK, *J. Biol. Chem.*, 179 (1949) 569.
[3] D. E. KOSHLAND, JR. AND M. J. ERWIN, *J. Am. Chem. Soc.*, 79 (1957) 2657.
[4] B. S. HARTLEY, M. A. NAUGHTON AND F. SANGER, *Biochim. Biophys. Acta*, 34 (1959) 244.
[5] M. NAUGHTON, F. SANGER, B. S. HARTLEY, D. S. SHAW, *Biochem. J.*, in the press.
[6] D. E. KOSHLAND JR., W. J. RAY AND J. M. ERWIN, *Federation Proc.*, 17 (1958) 1147.
[7] L. ANDERSON AND G. R. JOLLÈS, *Arch. Biochem. Biophys.*, 70 (1957) 121.
[8] E. A. BARNARD AND W. D. STEIN, *Advances in Enzymol.*, 20 (1958) 51.

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Characterisation of two malic dehydrogenases from rat liver

Differential centrifugation of a rat-liver homogenate has shown that malic dehydrogenase activity is located in both the mitochondrial and supernatant fractions[1]. The mitochondrial and supernatant enzymes have been shown to differ in their oxaloacetate-reduction kinetics[2], and in their electrophoretic mobilities[3]. In the present work a separation and partial purification of the two enzymes has been achieved, and some further comparisons made.

The rat livers were homogenised in 0.25 *M* sucrose and centrifuged. The 600–3,000 × *g* (15 min) precipitate and the 10,000 × *g* (30 min) supernatant were retained, as "mitochondrial" and "supernatant" fractions respectively. To release malic dehydrogenase from the mitochondria, an acetone powder was made, and this powder extracted with 0.05 *M* sodium phosphate, pH 7.0. Use of $(NH_4)_2SO_4$ fractionation, calcium phosphate adsorption and elution, and chromatography on Amberlite IRC 50 resin and DEAE-cellulose effected a 100-fold purification of the mitochondrial and a 250-fold purification of the supernatant enzyme. The two enzymes differ significantly in their chromatographic behaviour. The mitochondrial enzyme is retarded, and the supernatant enzyme unretarded by the Amberlite resin, whereas the reverse is true of the DEAE-cellulose (Fig. 1). The elution patterns are reproducible and characteristic, whether the enzymes are run separately or as a mixture, or when a crude rat-liver homogenate is tested. Pig-heart mitochondrial malic dehydrogenase, prepared by a modification of the method of Wolfe and Neilands[4], showed the same elution patterns as the rat-liver mitochondrial enzyme.

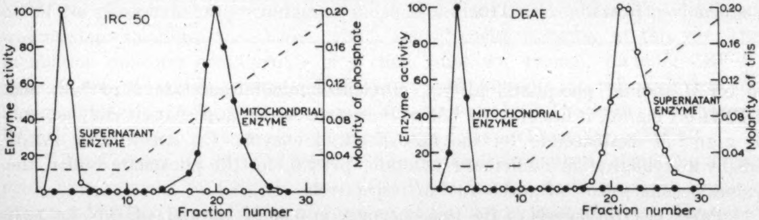

Fig. 1. Chromatography of rat-liver malic dehydrogenases on Amberlite IRC 50 and on DEAE-cellulose. Open circles, supernatant enzyme; solid circles, mitochondrial enzyme; broken line, molarity of eluting buffer (sodium phosphate, pH 7.0 for Amberlite; tris(hydroxymethyl)-aminomethane, pH 8.2 for DEAE-cellulose).

The substrate specificities of the enzymes were determined by comparing the rates of oxidation of malate and tartrate, and the rates of reduction of DPN and some of its analogues (a gift from Prof. N. O. Kaplan) (Table I).

Both rat-liver enzymes reduce oxaloacetate, the rate of reduction being affected by the inhibition due to high substrate concentration. A plot of reaction velocity against oxaloacetate concentration gives a curve with a maximum at a concentration which depends on both the pH and the nature of the buffer used. The position of the maximum, under defined conditions, distinguishes between the two enzymes;

Abbreviations: DEAE, diethylaminoethyl-; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; APDPN, acetylpyridine analogue of DPN; TNDPN, thionicotinamide analogue of DPN; DeDPN, deamino analogue of DPN.

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TABLE I

OXIDATION OF MALATE AND TARTRATE, AND REDUCTION OF DPN AND ITS ANALOGUES BY MALIC DEHYDROGENASES FROM DIFFERENT SOURCES

Reaction rates expressed as percentages of the rates with L-malate (20 mM) and DPN (1.2 mg/ml or 0.3 mg/ml, as shown); 0.1 M glycine–NaOH buffer, pH 10.0 (*cf.* ref. 4) used throughout.

Substrate		*Coenzyme*	*Enzyme source*		
			Rat-liver supernatant	*Rat-liver mitochondria*	*Pig-heart mitochondria*
		(1.2 mg/ml)			
L-malate	20 mM	DPN	100	100	100
L-tartrate	100 mM	DPN	0.16	1.54	1.79
meso-tartrate	50 mM	DPN	0.15	1.05	1.01
		(0.3 mg/ml)			
L-malate	20 mM	DPN	100	100	100
L-malate	133 mM	DPN	86	104	103
L-malate	20 mM	APDPN	83	105	90
L-malate	133 mM	APDPN	38	66	56
L-malate	20 mM	DeDPN	41	65	69
L-malate	20 mM	TNDPN	42	16	16
L-malate	20 mM	TPN	0	0	0

in 0.1 M sodium phosphate, pH 7.5, ethylenediaminetetraacetate $2 \cdot 10^{-3}$ M, and DPNH 0.1 mg/ml, it lies at $5 \cdot 10^{-4}$ M oxaloacetate for the supernatant enzyme, and at $2 \cdot 10^{-4}$ M oxaloacetate for the mitochondrial enzyme. On substituting 0.1 M tris(hydroxymethyl)aminomethane chloride, pH 7.6, for the phosphate buffer, the values become $5 \cdot 10^{-4}$ M and $6 \cdot 10^{-5}$ M respectively.

Paper electrophoresis of the two enzymes, in 0.06 M veronal, pH 8.6, for 12 h at 10 V/cm, 4°, confirmed (see ref. 3) that the supernatant enzyme moves toward the anode (5 cm under the conditions used), whereas the mitochondrial enzyme remains at the origin. Pig-heart mitochondrial malic dehydrogenase also remains at the origin.

The malic dehydrogenases of the rat-liver supernatant and of rat-liver mitochondria differ, then, in a number of respects, the latter resembling closely the enzyme of pig-heart mitochondria. Malic dehydrogenases from other sources are currently being investigated.

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[1] G. S. CHRISTIE AND J. D. JUDAH, *Proc. Roy. Soc. (London)*, *B* 141 (1953) 420.
[2] A. DELBRUCK, H. SCHIMASSEK, K. BARTSCH AND T. BUCHER, *Biochem. Z.*, 331 (1959) 297.
[3] T. WIELAND, G. PFLEIDERER, I. HAUPT AND W. WORNER, *Biochem. Z.*, 332 (1959) 1.
[4] R. G. WOLFE AND J. B. NEILANDS, *J. Biol. Chem.*, 221 (1956) 61.

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The action of carboxypeptidases A and B on the separated α and β chains of normal adult human hemoglobin

The molecule of normal adult human hemoglobin is known to be composed of four polypeptide chains[1], a pair of so-called α chains and a pair of β chains. The recently reported[2] clear-cut separation of these chains by countercurrent distribution or by chromatography[3] now makes possible reliable amino acid sequence studies. As part of a general study[4] of the splitting of the separated chains by enzymes, the action of carboxypeptidases A[5] and B[6,7] is particularly interesting and timely.

The α and β chains* were isolated by countercurrent distribution[2] of normal adult human globin prepared according to TEALE[8]. Carboxypeptidase-A was the Sigma Chemical Co. preparation, a 5-times crystallized toluol–water suspension. Its concentration was assayed spectrophotometrically assuming an ε_m of $8.6 \cdot 10^{-4}$ (ref. 5). Prior to use, this enzyme was treated with DFP, by adding 10 mg Na_2CO_3 to 1.00 ml of the enzyme suspension, and then a 200-fold molar excess of DFP. Carboxypeptidase-B was prepared from acetone powders of bovine pancreas** and activated according to FOLK AND GLADNER[6,7]. The NaCl extracts of procarboxypeptidase B (2.5 g acetone powder gives 2.00 ml of NaCl extract) had an activity of 1,200 units/ml, measured by hydrolysis of hippuryl-lysine. The reactions were carried out at 25°, pH 7.65, in a 0.2 *M* Na phosphate buffer containing 0.056 *M* sodium lauryl sulfate. This detergent was used to solubilize the chains, which are otherwise insoluble at this pH. The incubation mixtures contained: α or β chain (mol. wt. 17,000), 1.4 m*M*; CBP–B, 130 units/ml of reaction mixture; CBP–A, 0.1 m*M*. Aliquots (0.4 ml) were removed at time intervals and the reaction stopped by 0.2 ml 1 *N* HCl. The amino acids present in each aliquot were determined by quantitative column chromatography[9].

Table I shows the rates of release of amino acids from the α chain. No amino acids were released by CBP–A alone. CBP–B produced a rapid release of arginine associated with a rapid release of tyrosine and a slow release of lysine. The rapid

TABLE I

AMINO ACIDS RELEASED FROM THE α CHAIN IN μMOLES/μMOLE α CHAIN

CBP-B added initially; CBP-A added after 65 min.

Time (min)	*CBP–B*		*CBP–B + CBP–A*	
	35	*65*	*105*	*255*
Arginine	0.91	0.92	0.97	0.96
Tyrosine	0.86	0.86	0.85	0.89
Lysine	0.47	0.77	0.90	1.02
Serine	0.04	0.10	0.85	1.42
Threonine	—	0.03	0.35	1.40
Leucine	—	0.02	0.41	1.20
Valine	—	—	0.29	0.93
Alanine	—	—	0.15	0.28
Phenylalanine	—	—	0.12	0.14
Histidine	—	—	0.11	0.10

Abbreviations: CBP-A, carboxypeptidase A; CBP-B, carboxypeptidase B; DFP, diisopropylfluorophosphate.

* The chains were kindly furnished by Dr. R. J. HILL.

** The powder was kindly supplied by Dr. J. E. FOLK.

release of tyrosine, even before the addition of CBP–A, was probably caused by the known contamination of CBP–B preparations by small amounts of CBP–A. After addition of CBP–A, serine was released at the most rapid rate. The data indicate the following sequence for the C-terminal residues of the α chain: –Ser–Lys–Tyr–Arg. This result is confirmed by the work of HILL AND KONIGSBERG[4] who have found a Tyr–Arg peptide on tryptic hydrolysis of the α chain.

Table II shows the rates of release of amino acids from the β chain. The only amino acids released by CBP–A were histidine, tyrosine, and a small amount of lysine,

TABLE II

AMINO ACIDS RELEASED FROM THE β CHAIN IN μMOLES/μMOLE β CHAIN

CBP-A added initially; CBP-B added after 75 min.

Time (min)	*CBP–A*	*CBP–B + CBP–A*		
	75	*110*	*140*	*330*
Histidine	1.12	1.80	1.84	2.10
Tyrosine	0.92	0.95	0.96	0.96
Lysine	0.08	0.92	1.08	1.38
Alanine	0.02	0.64	1.67	4.55
Leucine	—	0.28	0.80	1.32
Serine	—	0.10	0.26	1.92
Valine	—	0.03	0.08	1.80
Glycine	—	—	—	0.45
Threonine	—	—	0.07	0.25
Phenylalanine	—	—	—	0.14

the latter probably by the CBP–B which contaminates CBP–A preparations. After addition of CBP–B, a rapid release of lysine was observed, accompanied by a concomitant release of histidine and alanine, at an equal rate. These data, together with other experiments done in this laboratory which show that histidine is the first amino acid released by CBP–A, indicate the following sequence for the β chain: –(His,Ala)–Lys–Tyr–His. This sequence is concordant with the observations of previous investigators, who have shown that histidine is released by hydrazinolysis and by CBP–A from both the β chain[10] and whole hemoglobin[11–13], and who have suggested that a Tyr–His peptide might be at the C-terminal position[10, 11].

The data for the other amino acids shown in Tables I and II do not allow any further conclusions to be drawn regarding the C-terminal sequence of the chains, because the rates of release of amino acids which are located several residues away from the C-terminal positions are complicated by the non-uniformity of enzymic digestion at these distant sites.

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[1] H. S. RHINESMITH, W. A. SCHROEDER AND L. PAULING, *J. Am. Chem. Soc.*, 79 (1957) 4782.
[2] R. J. HILL AND L. C. CRAIG, *J. Am. Chem. Soc.*, 81 (1959) 2272.
[3] V. M. INGRAM, *Nature*, 183 (1959) 1795.
[4] R. J. HILL AND W. KONIGSBERG, *J. Biol. Chem.*, in the press.

[5] H. Neurath, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 77.
[6] J. A. Gladner and J. E. Folk, *J. Biol. Chem.*, 231 (1958) 379.
[7] J. A. Gladner and J. E. Folk, *J. Biol. Chem.*, 231 (1958) 393.
[8] F. W. J. Teale, *Biochim. Biophys. Acta*, 32 (1959) 543.
[9] S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
[10] V. K. Hilse and G. Braunitzer, *Z. Naturforsch.*, 14b (1959) 603.
[11] H. A. Itano, *Advances in Protein Chemistry*, 12 (1957) 240.
[12] T. Kauffman and F. Boettcher, *Chem. Ber.*, 92 (1959) 2707.
[13] T. H. J. Huisman and A. Dozy, *Biochim. Biophys. Acta*, 20 (1956) 400.

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An effect of chloramphenicol on the incorporation of tritiated thymidine into chromosomal DNA

The inhibitive action of chloramphenicol on protein synthesis has been utilized extensively and with considerable success for the elucidation of the relationships between nucleic acids and proteins in microorganisms[1–3]. The apparent absence of a direct effect of this antibiotic on DNA replication in microorganisms[3], the probability that DNA in microorganisms is less intimately associated with a protein moiety than is chromosomal DNA[4,5], and the linear pattern of DNA increase in bacteria[6,7,8] as opposed to the stepwise pattern characteristic of DNA synthesis in chromosomal systems, have led us to the notion that chromosomal protein may play a controlling role in chromosomal DNA replication in addition to the structural function recently suggested[9,10]. We have explored this possibility by studying the incorporation of tritiated thymidine into meristematic cells of onion root tips in the presence and absence of chloramphenicol; the incorporation of $[^{14}\text{C}]$thymidine was studied under similar experimental conditions as a control for possible tritium effects[11].

Onion roots were immersed in a solution of 300 μg/ml chloramphenicol for periods of 6 and 24 h prior to the addition of labeled thymidine, with renewal of the chloramphenicol solutions every 6 h. $[^{3}\text{H}]$thymidine was used at a concentration of 2.5 μC/ml and a specific activity of 360 μC/μmole. The roots were incubated with the isotope for 11 h, fixed, briefly hydrolyzed, and squashed. Kodak AR-10 stripping film was applied and the preparations were exposed for 7 days. Assay of the $[^{3}\text{H}]$thymidine solutions before and after incorporation indicated that ample amounts of the tracer were available for incorporation throughout the incubation period.

The semi-quantitative analysis of the $[^{3}\text{H}]$thymidine-labeled preparations led to the following conclusions: (a) the percentage of cells with labeled nuclei did not differ significantly from control values in the root tips treated with chloramphenicol for either 6 h or 24 h; (b) the percentage of heavily labeled nuclei (nuclei with autoradiographic grain densities above the limit for reliable direct counting) was considerably higher in the treated roots than in controls. Instrumental analysis of the heavily labeled fraction of nuclei by means of a scanning microscope designed by Gullberg[12] resulted in the data plotted in Fig. 1. The graph shows a progressive increase in the amount of $[^{3}\text{H}]$thymidine incorporation in nuclei of chloramphenicol-treated roots. The effect of the drug appears even more strikingly when we consider that the heavily

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Abbreviation: DNA, deoxyribonucleic acid.

labeled fraction represents a larger proportion of the cells in treated than in control roots. Photometric DNA measurements showed an increase in DNA levels which could account for some, if not all, of the increased [^{3}H]thymidine incorporation in the presence of chloramphenicol. Further details of this analysis will be published elsewhere.

Very different results were obtained when [^{14}C]thymidine (0.5 μC/ml, 2.7 μC/mole) was substituted for the tritiated precursor: no significant incorporation differences were observed between either of the two chloramphenicol treatments and the controls. While the [^{3}H]thymidine-incorporation data thus show a chloramphenicol effect which suggests a functional role for protein in chromosomal DNA synthesis, the [^{14}C]thymidine results indicate that we are dealing primarily with an effect of tritiated thymidine on DNA which is amplified in the presence of chloramphenicol.

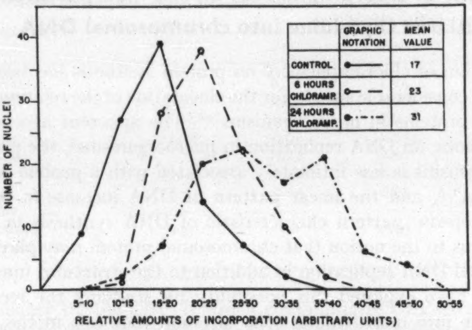

Fig. 1. The incorporation of [^{3}H]thymidine into meristematic nuclei of onion roots under the influence of chloramphenicol (300 μg/ml).

Further experiments, guided by the working hypothesis that normal chromosomal DNA replication requires the integrity of the DNA molecule as well as the presence of a proteinaceous stabilizing element, are in progress.

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[1] T. D. BROCK AND M. L. BROCK, *Biochim. Biophys. Acta*, 33 (1959) 274.
[2] M. NOMURA AND J. D. WATSON, *J. Mol. Biol.*, 1 (1959) 104.
[3] J. TOMIZAWA, *Virology*, 6 (1958) 55.
[4] G. ZUBAY AND M. R. WATSON, *J. Biophys. Biochem. Cytology*, 5 (1959) 51.
[5] M. H. F. WILKINS AND G. ZUBAY, *J. Biophys. Biochem. Cytology*, 5 (1959) 55.
[6] E. MCFALL AND G. S. STENT, *Biochim. Biophys. Acta*, 34 (1959) 580.
[7] M. SCHAECHTER, M. W. BENTZON AND O. MAALOE, *Nature*, 183 (1959) 1207.
[8] I. E. YOUNG AND P. C. FITZ-JAMES, *Nature*, 183 (1959) 372.
[9] M. H. F. WILKINS AND G. ZUBAY, *J. Mol. Biol.*, 1 (1959) 1.
[10] G. ZUBAY AND P. DOTY, *J. Mol. Biol.*, 1 (1959) 179.
[11] M. KRAUSE AND W. PLAUT, *Nature*, in the press.
[12] J. E. GULLBERG, *Lab. Investigations*, 8 (1959) 94.

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Disturbance of RNA turnover in the cell nucleus by X-irradiation[*] in the early phase of rat-liver regeneration

Several investigators[1–3] have shown that a rather low dose of total-body X-radiation could give a delay of DNA synthesis and mitosis in regenerating liver, if that dose was administered before DNA synthesis had started, *i.e.* in the so-called presynthetic period. In case of regeneration after partial hepatectomy this presynthetic period ends 15–18 h after operation[4,5] and is followed by a period of accelerated DNA synthesis with a maximum at about 24–30 h after operation. The first mitoses are seen at about 22–24 h after operation. The same low radiation dose given in the period of DNA synthesis had no immediate effect on the incorporation of precursors in DNA but could delay the onset of mitotic activity[2].

Preliminary results[6] of investigations on the effect of total-body X-irradiation on the metabolism of nuclear RNA in resting liver prompted us to study the sensitivity of nuclear RNA metabolism in the presynthetic period of liver regeneration after partial hepatectomy.

RNA turnover in the nucleus was studied by following the incorporation rate of ^{32}P between 0 and 28 h after operation. Seven groups of 4 male WAG-rats (about 200 g) were hepatectomized as described by HIGGINS AND ANDERSON[7]; at 0, 4, 8, 12, 16, 20 or 24 h after operation 200 μC ^{32}P (in 0.5 ml physiological saline containing 0.1 % Na_2HPO_4) were injected intravenously into each animal and 4 h later the remaining liver lobes of each group were pooled and analyzed by standard methods for the determination of the specific activity of DNA and of the mononucleotides of nuclear RNA.

The results of this experiment are shown in Fig. 1. The start of DNA synthesis

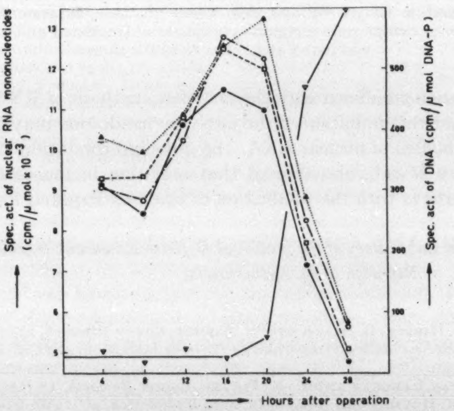

Fig. 1. Incorporation of ^{32}P in DNA and the monopnucleotides of nuclear RNA at various intervals after partial hepatectomy. ●—●, GMP; ○-·-·-○, UMP; □----□, CMP; ▲·····▲, AMP; ▽—▽, DNA.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; CMP, cytidine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate.

coincides with the maximum of RNA turnover and it seems that synthesis of RNA must precede that of DNA.

It was found that irradiation with 700 R 12 h after partial hepatectomy resulted in a slight increase of the specific activity of nuclear RNA 4 h later. In subsequent experiments the irradiation was therefore carried out at shorter time intervals after operation, *i.e.* at 2, 4 and 6 h; moreover, nuclear RNA was fractionated into a fraction extractable from the nuclei with 1 *M* NaCl (n-RNA I) and a fraction not extractable with 1 *M* NaCl (n-RNA II).

The results of the irradiation experiments at short time intervals after operation are summarized in Table I. They show clearly that incorporation of ^{32}P into nuclear RNA is inhibited by ionizing radiation in the very early phase of liver regeneration.

TABLE I

DEPRESSION OF INCORPORATION OF ^{32}P INTO RNA OF RAT LIVER NUCLEI BY X-IRRADIATION IN THE EARLY PHASE OF REGENERATION AFTER PARTIAL HEPATECTOMY

Time of irradiation (h after operation)	*RNA fraction*	*Depression of specific activity in percent of the control for*			
		CMP	*AMP*	*GMP*	*UMP*
2	n-RNA I	40	29	40	41
2	n-RNA II	43	63	38	39
4	n-RNA I	50	58	54	57
4	n-RNA II	47	42	33	—
6	n-RNA I	24	20	17	9
6	n-RNA II	no depression			

6 groups of 4 animals were partially hepatectomized; 3 groups were irradiated with 700 R at respectively 2, 4 and 6 h after operation. Control and irradiated animals received at respectively 4, 6 and 8 h after operation 200 μC ^{32}P and were killed 2 h later. In one of these experiments the specific activities of cytoplasmic inorganic phosphate of irradiated and control animals were compared and found to be the same.

This observation combined with the fact that synthesis of RNA precedes that of DNA suggests that the inhibition of the latter by irradiation may be due to disturbance of the metabolism of nuclear RNA. The same interpretation is borne out by the results of BOLLUM *et al.*[8], who showed that radiation in the early stages of liver regeneration interferes with the production of enzymes required for DNA synthesis.

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[1] L. S. KELLY, J. D. HIRSCH, G. BEACH AND W. PALMER, *Cancer Research*, 17 (1957) 117.
[2] B. E. HOLMES, *Ciba Foundation Symposium on Ionizing Radiations and Cell Metabolism*, London, 1956, p. 225.
[3] R. E. BELTZ, J. VAN LANCKER AND V. R. POTTER, *Cancer Research*, 17 (1957) 688.
[4] D. B. CATER, B. E. HOLMES AND L. K. MEE, *Acta Radiologica*, 46 (1956) 655.
[5] L. I. HECHT AND V. R. POTTER, *Cancer Research*, 16 (1956) 988.
[6] H. M. KLOUWEN, personal communication.
[7] G. M. HIGGINS AND R. M. ANDERSON, *Arch. Pathol.* 12 (1931) 186.
[8] F. J. BOLLUM, J. W. ANDEREGG, A. B. MCELYA AND V. R. POTTER, *Cancer Research*, 20 (1960) 138.

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Studies concerning inhibition of the synthesis of deoxycytidine by phosphorylated derivatives of thymidine

As described for other mammalian cells[1,2] the murine neoplastic cell lines, L5178Y and P815Y, require thymidine for reproduction in culture if synthesis *de novo* of this compound is inhibited with either 5-fluoro-2′-deoxyuridine or amethopterin. Under these conditions, and with successive increases in the concentration of thymidine in the medium, cellular reproduction is increased progressively until the extent of growth of the cells equals or even surpasses that of the controls; however, further increments in the amounts of added thymidine progressively inhibit growth (Fig. 1). The inhibitory phases of this response can be demonstrated equally well in the absence of either antimetabolite.

The recent observation that the triphosphate of either thymidine, deoxyadenosine or deoxyguanosine inhibits the conversion of the ribonucleotide, cytidine 5′-phosphate to the corresponding 2′-deoxy compound[4] suggested that the inhibition of growth by thymidine might be accounted for in a similar manner, and a study of the effect of 2′-deoxyribo nucleosides on the toxicity of thymidine was indicated. 2′-Deoxycytidine alone at low levels was found to prevent thymidine inhibition (Fig. 1). However, 2′-deoxyuridine, 2′-deoxyadenosine or 2′-deoxyguanosine, either singly or in combinations at $5 \cdot 10^{-4}$ *M* and at $5 \cdot 10^{-5}$ *M* were inactive. In the presence of an otherwise inhibitory high concentration of thymidine ($1.8 \cdot 10^{-3}$ *M*), half-maximal growth is obtained by the addition of a low concentration of 2′-deoxycytidine of the same order as that required with thymidine ($1 \cdot 10^{-6}$ *M*) for growth in the presence of the abovementioned antimetabolites (see Fig. 2). Cytidine, even at a concentration of $4 \cdot 10^{-4}$ *M*, has little or no effect on the inhibition of growth by thymidine.

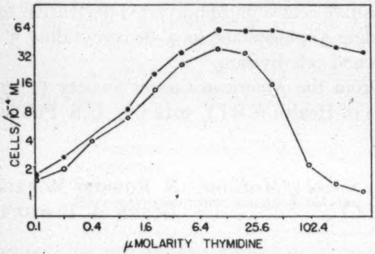

Fig. 1. The support and subsequent inhibition of growth by thymidine in the presence of $4 \cdot 10^{-8}$ *M* FUDR (40 times the 50% inhibitory level). Culture techniques and media are similar to those described by FISCHER[3]. Tubes were inoculated with 10^4 cells/ml medium and the number of cells was determined, using a Coulter counter, after incubation for 72 h. 2′-Deoxycytidine, to give a final concentration of $4 \cdot 10^{-5}$ *M*, was added to the tubes represented by closed circles. None was added to those tubes represented by open circles.

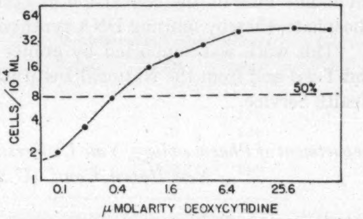

Fig. 2. Reversal by 2′-deoxycytidine of thymidine-induced inhibition of reproduction of L5178Y cells. Each tube contained $1.8 \cdot 10^{-3}$ *M* thymidine plus 2′-deoxycytidine as noted; 50% growth was supported by $6 \cdot 10^{-7}$ *M* 2′-deoxycytidine. Similar results were obtained with P815Y cells and 50% growth was supported by $6 \cdot 10^{-6}$ *M* 2′-deoxycytidine.

Presumably thymidine inhibits growth only after its conversion to the 5′-

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Abbreviation: FUDR, 5-fluoro-2′-deoxyuridine.

TABLE I

RATE OF PHOSPHORYLATION OF THYMIDINE BY EXTRACTS OF FUDR-SENSITIVE AND FUDR-RESISTANT MAST CELLS

0.25 ml of supernatant (100,000 × g for 1 h) was added to 1.0 ml of a mixture compounded such that these final concentrations were obtained: $8\cdot10^{-5}$ M [^{3}H]thymidine (9 μC/μmole), $4.2\cdot10^{-3}$ M ATP, $6\cdot10^{-3}$ M 3-phosphoglycerate, $4.8\cdot10^{-3}$ M Mg^{++} and $1.25\cdot10^{-1}$ M tris(hydroxymethyl)-aminomethane, pH 8.0. Incubations were carried out at 36° and terminated by chilling and addition of 0.1 ml 70% $HClO_4$. $KClO_4$ was precipitated prior to the separation of phosphorylated compounds from thymidine using Dowex 1 × 10 formate[6]. Activity is expressed as moles product/min/mg protein.

Sensitive (P815Y)	*Resistant (P815Y/FUDR)*
$1\cdot10^{-10}$	$3\cdot10^{-12}$

phosphate, since a strain of P815Y selected for high resistance to FUDR[9], which is apparently deficient in thymidine kinase activity (Table I), is not inhibited by thymidine[5]. The results suggest that in sensitive cells a phosphorylated derivative of thymidine inhibits the conversion of cytidine 5′-phosphate to 2′-deoxycytidine 5′-phosphate, thereby limiting DNA synthesis and cell division.

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[1] M. A. Rich, J. L. Bolaffi, J. E. Knoll, L. Cheong, M. L. Eidinoff, *Cancer Research*, 18 (1958) 730.
[2] M. T. Hakala and E. Taylor, *J. Biol. Chem.*, 234 (1959) 126.
[3] G. A. Fischer, *Proc. N.Y. Acad. Sci.*, 76 (1958) 673.
[4] P. Reichard, Z. N. Canellakis and E. S. Canellakis, *Biochim. Biophys. Acta*, in the press
[5] N. R. Morris and G. A. Fischer, in preparation.
[6] F. J. Bollum and V. R. Potter, *Cancer Research*, 19 (1959) 561.

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Biological oxidation of N,N-dialkyl carbamates

N-Alkyl and N,N-dialkyl carbamates are widely used as insecticides [1,2] and medicinals for the relief of myasthenia gravis and other disorders[3]. The ultimate mode of action appears, in both cases, to be the competitive inhibition of cholinesterase. Cholinesterase[4] and plasma albumin[2,5] have been implicated in the biological degradation of N-alkyl carbamates. N,N-dimethyl carbamoyl fluoride is hydrolyzed by the plasma A-esterase and certain other esterases in the rabbit[6]. The N,N-dialkyl carba-

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Abbreviations: DpNC, N,N-dimethyl p-nitrophenyl carbamate; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; SKF 525-A, β-diethylaminoethyl diphenylpropylacetate. The chemical designations for insecticides and synergists are given in papers cited[1,2,10,11].

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mates derived from aromatic and heterocylic enols are not degraded by these mechanisms[2]. It appeared of interest to examine alternate pathways for metabolism of these compounds.

DpNC was used for preliminary studies because of the ease of analysis for degradation products. The *p*-nitrophenate ion was determined directly at 400 mμ and formaldehyde by the chromotropic acid method[7]. It was found that DpNC was attacked by an enzyme system in rat-liver microsomes requiring TPNH and oxygen. If the liver soluble fraction was present, TPN could be substituted for TPNH (Table I).

TABLE I

LOCALIZATION OF DpNC-DEGRADING ACTIVITY IN SUBCELLULAR FRACTIONS FROM RAT LIVER

Microsomes + soluble was the supernatant fraction ($S_{15\,000}$) after centrifugation of a 20% rat-liver homogenate in potassium phosphate buffer (pH 7.0, 0.05 *M*) at 15,000 × *g* for 30 min. Microsomes were the residue, and soluble the supernatant after centrifugation of $S_{15,000}$ at 105,000 × *g* for 1 h. Microsomes were resuspended in phosphate buffer. DpNC was added first to the flasks in ethanol solution and the ethanol evaporated. Flasks contained 1.0 mg DpNC, 3.7 mg co-factor, 1.5 ml enzyme preparation, 1.0 ml buffer and distilled water to a total volume of 5.0 ml. After 2-h incubation at 37° with shaking in air, the *p*-nitrophenol from subsequent degradation of the metabolite at pH 11.0 was measured.

Fraction	*p-nitrophenol with cofactor indicated*		
	None	*TPN*	*TPNH*
Microsomes + soluble	15	94	120
Microsomes	0	16	120
Soluble	5	11	11
Reconstituted microsomes + soluble	7	120	120

This resembles the N-dealkylation systems which have been described for several drugs[8], but differs in that the methyl group is not liberated as free formaldehyde under the conditions of incubation. A material is formed, however, which yields formaldehyde under strong acid conditions and can be completely extracted into ether from aqueous solution. Free formaldehyde cannot be extracted in this manner. During enzymic oxidation no free *p*-nitrophenol is formed. The metabolite has a hydrolytic half-life of 37 min at pH 8.0 based on liberation of *p*-nitrophenol, which differentiates it from DpNC and N-methyl *p*-nitrophenyl carbamate which have half-lives of over one year and less than 1 min respectively under the same conditions.

The product from DpNC, as analyzed with or without ether extraction, yielded a 1:1 ratio of formaldehyde: *p*-nitrophenol on degradation. A similar product from N-methyl N-ethyl *p*-nitrophenyl carbamate also yielded a 1:1 ratio indicating that only one methyl group is attacked. This same metabolite was demonstrated in the liver of rats 4 h after oral administration of DpNC. By analogy with the mechanism proposed for N,N-dimethyl tryptophane metabolism[9], it appears that the metabolite might be N-methyl N-methylol *p*-nitrophenyl carbamate. The microsome preparation also degraded the higher N,N-dialkyl *p*-nitrophenyl carbamates, the activity decreasing with increasing chain length (N,N-dimethyl > N,N-diethyl > N,N-di-*n*-propyl > N,N-di-isopropyl = N,N-dibutyl). The metabolites formed were less stable to alkaline hydrolysis than that from the dimethyl compound, even though the original compounds are more stable[2].

The microsomal oxidation of DpNC and its N,N-diethyl analog were inhibited by $1 \cdot 10^{-4}$ M SKF 525-A, piperonyl butoxide and sesamex. Determination of the effective inhibitor levels was difficult owing to the insolubility of the substrates and the methylene dioxyphenyl compounds (piperonyl butoxide and sesamex). It is interesting to note that the two methylene dioxyphenyl compounds are effective synergists of the toxicity to house flies for certain N,N-dimethyl carbamates[10], and that toxicity studies with other insecticides and house flies have led to the hypothesis that these synergists may inhibit oxidative detoxification mechanisms[11]. Administration of DpNC to house flies yielded a metabolite which appeared to be the same as that formed in rats or by isolated liver microsomes.

45 N,N-dimethyl carbamates or closely related compounds were incubated with the liver soluble plus microsome fraction with or without TPN fortification to determine the structural specificity for oxidation. Many carbamates including the insecticides Dimetan, Dimetilan, Isolan, Pyramat and Pyrolan and the parasympathomimetic agents neostigmine and physostigmine were metabolized to formaldehyde-yielding derivatives. The formaldehyde yield was markedly increased by the addition of TPN. When the change in anticholinesterase activity during incubation was followed, it was found that Isolan and certain other compounds were rapidly destroyed. However, the loss of anticholinesterase activity did not always correspond with the ability to yield formaldehyde, indicating that certain of the intermediates may themselves be anticholinesterase agents.

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[1] R. L. METCALF, *Organic Insecticides; Their Chemistry and Mode of Action*, Interscience Publ. Inc., N.Y., 1955, p. 392.
[2] J. E. CASIDA, K-B. AUGUSTINSSON AND G. JONSSON, *J. Econ. Entomol.*, 53 (1960) 205.
[3] A. STEMPEL AND J. A. AESCHLIMANN, Synthetic analogs of physostigmine, in F. F. BLICKE AND R. H. COX, *Medicinal Chemistry*, Vol. III (1956), Chap. 4, pp. 238, John Wiley & Sons Inc., N.Y.
[4] A. GOLDSTEIN AND R. E. HAMLISCH, *Arch. Biochem. Biophys.*, 35 (1952) 12.
[5] J. E. CASIDA AND K-B. AUGUSTINSSON, *Biochim. Biophys. Acta*, 36 (1959) 411.
[6] K-B. AUGUSTINSSON and J. E. CASIDA, *Biochem. Pharmacol.*, 3 (1959) 60.
[7] W. R. FRISELL, L. A. MEECH AND C. G. MACKENZIE, *J. Biol. Chem.*, 207 (1954) 709.
[8] B. B. BRODIE, J. R. GILLETTE AND B. N. LA DU, *Ann. Rev. Biochem.*, 27 (1958) 427.
[9] M. S. FISH, C. C. SWEELEY, N. M. JOHNSON, E. P. LAWRENCE AND E. C. HORNING, *Biochim. Biophys. Acta*, 21 (1956) 196.
[10] M. E. ELDEFRAWI, R. MISKUS AND V. SUTCHER, *J. Econ. Entomol.*, 53 (1960) 231.
[11] Y-P. SUN AND E. R. JOHNSON, *J. Agr. Food Chem.*, 8 (1960) in the press.

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Effect of anaerobiosis and addition of keto acids on glutamine utilization by Ehrlich ascites-tumor cells

Ehrlich ascites-tumor cells metabolize glutamine rapidly with the formation of equivalent amounts of ammonia[1]. Anaerobiosis produced by partial vacuum or by nitrogen reduced glutamine utilization considerably. This effect has been studied and related to the effect of keto acids.

It was found that anaerobiosis did not irreversibly reduce glutamine utilization; glutamine disappearance and ammonia formation were markedly stimulated by air after pre-incubation for 1 h in N_2. It was also found that malonate (0.06 *M*) and 2,4-dinitrophenol (10^{-4} *M*) did not affect glutamine uptake appreciably. This suggested that glutamine uptake was not dependent upon intact oxidative phosphorylation. No significant L-glutamine oxidase activity could be detected in homogenates of ascites cells, and glutamine exerted no effect on O_2 uptake and CO_2 evolution by whole cells. The effect of anaerobiosis on glutamine uptake was also demonstrated in cell homogenates and in suspensions of mitochondria fortified with adenosine triphosphate. Thus the reduced anaerobic glutamine uptake was not explained by lower anaerobic permeability of the cell membrane for glutamine.

Glutamine which disappeared under anaerobic condition accumulated almost quantitatively as glutamate. Under aerobic conditions the glutamine taken up could be largely accounted for as glutamate and aspartate, but some alanine was also found. This suggested that the increased glutamine utilization under aerobic conditions was due to transaminase reactions.

Aspartate, alanine and glutamate were determined by quantitative paper chromatography, using a modification of REDFIELD's technique[2]. In addition, glutamine, glutamate and α-ketoglutarate were estimated enzymically[3,4].

In further experiments, it was found that oxaloacetate markedly stimulated the anaerobic glutamine utilization with the formation of equivalent amounts of α-Ketoglutarate and aspartate, plus some alanine (Table I). Pyruvate only slightly stimulated glutamine utilization, and α-ketoglutarate and alanine were found. α-ketoglutarate exerted no effect on glutamine utilization. Oxaloacetate also stimulated glutamine utilization by isolated mitochondria both under aerobic and anaerobic conditions. Oxaloacetate had little effect on glutamine utilization in the supernatant fraction of 20,000 × *g*, while pyruvate stimulated glutamine utilization almost to the same extent as in mitochondria. This suggested that two different transaminases were present, a mitochondrial bound transaminase involving oxaloacetate and a water-soluble transaminase involving pyruvate.

Thus, the results could either be explained by the combined action of glutamine transaminases and amidases or by the action of glutaminase I and glutamate transaminases. Glutaminase I as well as glutamate–aspartate transaminase and glutamate–alanine transaminase were also found in mitochondria in sufficient amounts to explain the effect of keto acids on glutamine utilization. However, oxaloacetate exerted no appreciable effect on the final glutamate concentration when glutamine was present; furthermore, the glutaminase I activity could be destroyed by heating cell homogenate for 10 min at 50°, but oxaloacetate still stimulated glutamine utilization. Therefore, it appears likely that ascites cells contained an active mitochondrial bound glutamine–aspartate transaminase and a less active water-soluble

TABLE I

THE EFFECT OF KETO ACIDS ON GLUTAMINE UTILIZATION

Washed ascites-tumor cells (0.8 ml packed cells), mitochondria (40 mg protein) and supernatant of 20,000 × g (50 mg protein) prepared from ascites-tumor cells, were incubated in air or in N_2. The N_2 had been washed in an alkaline pyrogallol solution. Additions (mM): L-glutamine, 8; potassium phosphate buffer pH 7.4, 10; tris(hydroxymethyl)aminomethane buffer pH 7.4, 10; where indicated, oxaloacetate, 25, pyruvate, 14, or α-ketoglutarate, 14 were added (sodium salts). Oxaloacetic acid was neutralized with Na_2CO_3 immediately before use. The final volume was 6 ml. The substrates were added after temperature equilibration for 10 min. The incubation was then continued for an additional 60 min. The whole cells were incubated at 37°, the cell fractions at 30°.

	Additions	*Gas phase*	*Glutamine disappearing (μmoles)*	*NH_3 (μmoles)*	*Glutamate (μmoles)*	*Aspartate (μmoles)*	*Alanine (μmoles)*	*α-keto-glutarate (μmoles)*
Whole cells	—	Air	31.4	36.0	14.8	10.9	8.4	0.2
	—	N_2	17.0	21.6	16.1	0.9	5.4	0.1
	Oxaloacetate	Air	38.5	41.3	15.8	14.5	11.5	5.0
	Oxaloacetate	N_2	48.0*	50.3	17.3	22.2	13.0	27.7
	Pyruvate	N_2	20.0	23.8	14.8	1.7	12.5	7.3
	α-ketoglutarate	N_2	16.2	20.3	17.5	1.2	6.0	—
Mitochondria	—	N_2	15.3	16.4	3.3	0	2.3	0
	Oxaloacetate	N_2	39.1	40.2	4.5	23.0	3.0	22.0
	Pyruvate	N_2	15.3	16.1	3.1	0	5.1	2.0
Supernatant	—	N_2	7.7	6.6	2.1	0	4.7	0
	Oxaloacetate	N_2	9.0	11.7	0.6	0	5.2	4.6
	Pyruvate	N_2	9.0	8.8	0.3	0	8.2	2.0

* Supply of glutamine exhausted.

glutamine–alanine transaminase. The latter enzyme might be identical with liver glutaminase II[5]. It has also been reported that oxaloacetate stimulates ammonia production from glutamine in liver extract, but the effect of oxaloacetate was somewhat lower than that of pyruvate, and both reactions occurred in the water-soluble fraction[6]. Thus this enzyme might be distinguished from that of ascites cells by its relative activity and solubility.

The results suggest that the reduced anaerobic glutamine utilization may be related to lack of oxaloacetate under anaerobic conditions.

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[1] E. KVAMME AND G. SVENNEBY, *4th Intern. Congr. Biochem., Vienna,* 1958, Suppl. Intern. Abstr. Biol. Sci., 6–67, p. 78.
[2] R. R. REDFIELD AND E. S. G. BARRON, *Arch. Biochem.*, 35 (1952) 443.
[3] H. A. KREBS, *Biochem. J.*, 43 (1948) 51.
[4] E. KVAMME, *Acta Physiol. Scand.*, 42 (1958) 231.
[5] J. P. GREENSTEIN AND F. M. LEUTHARDT, *Arch. Biochem.*, 17 (1948) 105.
[6] A. MEISTER AND S. V. TICE, *J. Biol. Chem.*, 187 (1950) 173.

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CHEMICAL INVESTIGATIONS OF THE GIANT NERVE FIBERS OF THE SQUID

III. IDENTIFICATION AND QUANTITATIVE ESTIMATION OF FREE ORGANIC NINHYDRIN-NEGATIVE CONSTITUENTS

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SUMMARY

In the dialyzable portion of axoplasm of *Loligo pealii* and *Dosidicus gigas* were identified and quantitatively estimated: glycocoll-betaine, glycerol and myo-inositol in the free state. The total of these three components, described for the first time in the axoplasm of both species of squid, amounts to approximately 18 % and 23 % respectively, on the dry weight basis of the material investigated.

Hypoxanthine and carbohydrates, are present in small quantities. It is surprising to find sucrose among the carbohydrates.

INTRODUCTION

In a program having as its aim an exhaustive chemical investigation of the constituents of the axoplasm of the giant nerve fiber of the squid we have thus far reported on the free amino acids[1] and on a substance previously not described in biological material, cysteic acid amide (β-sulfoalaninamide)[2,50]. The latter substance represents a third sulfonate found in relatively high concentrations in the axoplasm of *Loligo pealii* but in rather small amounts in *Dosidicus gigas* axoplasm, the other two being isethionic acid (2-hydroxyethane sulfonic acid) and taurine. Despite the presence of these sulfonates and of high concentrations of free amino acids, cysteine and cystine appear to be absent in the free state. Ornithine is present instead of histidine.

From the analytical results reported on the dialyzable constituents of squid axoplasm it appears that, in addition to certain constituents such as peptides (present in very low concentrations) ninhydrin-positive constituents represent about 30 % in *Loligo* and 25 % in *Dosidicus*. From the data of others[3-5], inorganic ions represent 22 % in *Loligo*. This leaves a deficit, made up of ninhydrin-negative substances, of 48 % in *Loligo* and probably somewhat higher in *Dosidicus* (51 %, assuming the same concentration of inorganic ions as in *Loligo*).

In the analytical investigation to be described in this paper the following substances have been identified for the first time in squid axoplasm, and some of them have been shown to be present in substantial concentrations: glycocoll-betaine, glycerol, myo-inositol, muscle adenylic acid (5'-AMP), homarine, hypoxanthine, sucrose, glucose and fructose*. These substances add up to 22 % of the dialyzable constituents of the axoplasm of *Loligo* and 26 % of that of *Dosidicus*. This reduces the unidentified material to less than 10 % as will be shown in the balance sheet given in the following paper[6].

All results pertain to lyophilized axoplasm which had been dialyzed against distilled water in the cold as previously described[1].

EXPERIMENTAL

Material and methods

The axoplasm used in these analyses was part of the same lyophilized and dialyzed material used for the estimation of free amino acids[1].

Separation into 11 fractions was accomplished by means of preparative paper electrophoresis at pH 3.9, using an acetic acid–pyridine buffer[7]. The resulting neutral fraction (N.F.$_{3.9}$) was fractionated at pH 2.4 with 0.5 *N* acetic acid as electrolyte, yielding 5 fractions, one neutral (N.F.$_{2.4}$), and four which were deflected with different mobilities toward the cathode. The methods of fractionation, visual demonstration and partial identification of the constituents were given previously[1]. The next step in the electrophoretic fractionation of N.F.$_{2.4}$ at pH 10.2 (using a tri-ethylamine–acetic acid buffer), mainly into a neutral migrating fraction (N.F.$_{10.2}$) and a fraction strongly deflected toward the anode, is described below.

RESULTS

With the exception of muscle adenylic acid, all the components herein described are contained in N.F.$_{3.9}$. By electrophoretic separation of this fraction at pH 2.4 betaine and hypoxanthine were isolated. Further electrophoretic separation of N.F.$_{2.4}$ at pH 10.2 freed this fraction of taurine and cysteic acid amide, which at this pH are deflected toward the anode, leaving the neutral constituents glycerol, myo-inositol, homarine and carbohydrates. Muscle adenylic acid is found in the region of glutamic acid after the electrophoresis at pH 3.9.

Diagram I shows the steps of electrophoretic separation; the substances in brackets are not considered in this article.

Table I represents the proportion by weight of the neutral fractions obtained by electrophoresis at pH 3.9, 2.4 and 10.2, respectively, based on dry weight.

The electrophoretic behavior of the components at different pH values together with their weights indicated some chemical characteristics of these substances and also reflected the degree of purity of the fractions. The use of easily volatilizable buffers for the electrophoresis is a prerequisite for a weight determination of such

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* The dialyzable organic constituents described thus far by us in squid axoplasm are, in the main, not unique for nerve but occur in varying concentrations also in squid blood. This was discovered by one of us (G.G. J.D.) soon after the initiation of a program of investigation of the constituents of squid blood. These constituents are present at a lower level of total concentration than in axoplasm. Among the free organic substances present in axoplasm but not yet demonstrated in squid blood are cysteic acid amide, isethionic acid and peptides[1,2]. The results of the detailed analyses will be communicated in due course. They are referred to here primarily because of their bearing on the possible physiological role of the dialyzable organic substances thus far demonstrated to occur in axoplasm.

DIAGRAM I

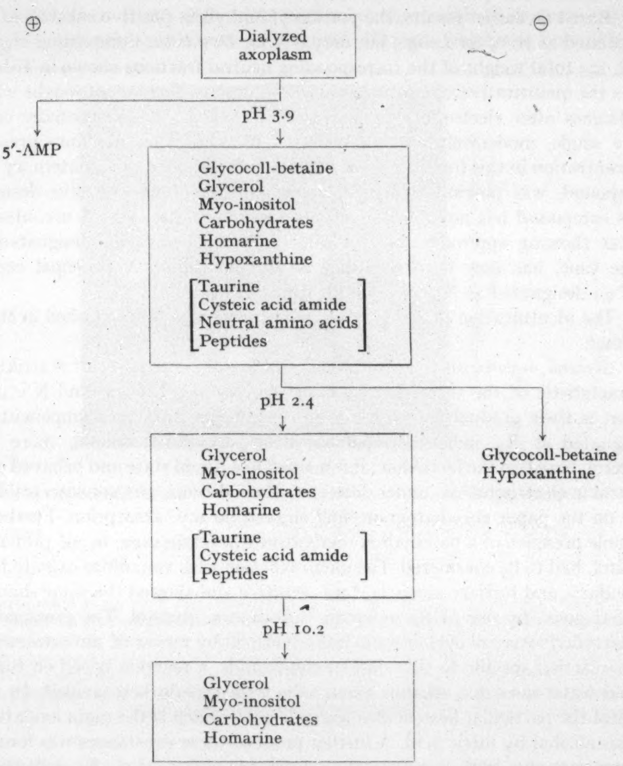

⊕
⊖
Dialyzed axoplasm
pH 3.9
5′-AMP
Glycocoll-betaine
Glycerol
Myo-inositol
Carbohydrates
Homarine
Hypoxanthine
Taurine
Cysteic acid amide
Neutral amino acids
Peptides
pH 2.4
Glycerol
Myo-inositol
Carbohydrates
Homarine
Taurine
Cysteic acid amide
Peptides
Glycocoll-betaine
Hypoxanthine
pH 10.2
Glycerol
Myo-inositol
Carbohydrates
Homarine

TABLE I

DISTRIBUTION BY WEIGHT OF THE ELECTROPHORETICALLY NEUTRAL FRACTIONS OF THE DIALYZED AXOPLASM

Fraction	*% dry weight*	
	Loligo	*Dosidicus*
N.F.$_{3.9}$	37·96	32·42
N.F.$_{2.4}$	25·34	14·82
N.F.$_{10.2}$	12·07	10·57

electrophoretically separated fractions. The final identification and quantitative estimation of their individual components was achieved by paper chromatography, specific color reactions on paper and in solution, observation of the behavior under u.v. light of the components being studied, and titration methods.

Identification and quantitative estimation

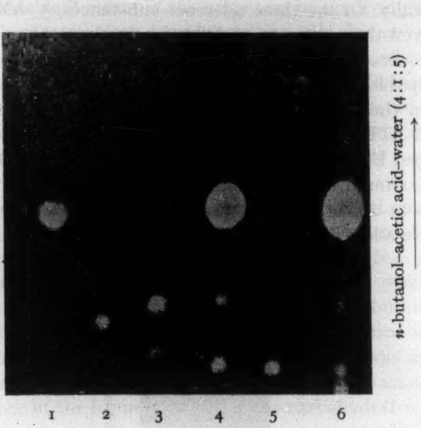
Based on earlier results, the portion of ninhydrin-positive substances in N.F.$_{3.9}$ is calculated as 16 % for *Loligo*, but only 6 % for *Dosidicus*. Comparison of these figures with the total weight of the corresponding neutral fractions shown in Table I emphasizes the quantitative significance of these fractions. The loss of weight which N.F.$_{3.9}$ undergoes after electrophoretic separation at pH 2.4 is substantially compensated by a single, moderately cathode-deflected fraction. The substance present in high concentration in this fraction, providing the color reaction of a quaternary ammonium compound, was previously demonstrated and given the tentative designation X_1. This compound has now been identified as glycocoll-betaine. A u.v.-absorbent substance showing approximately the same cathodal deflection, designated X_2 at the same time, has now been identified as hypoxanthine. A principal component of N.F.$_{2.4}$ designated as X_3 has been identified as glycerol.

The identification of the various constituents was accomplished in the following manner.

Glycerol, myo-inositol, carbohydrates and muscle adenylic acid: A striking physical characteristic of the three neutral fractions, N.F.$_{3.9}$, N.F.$_{2.4}$, and N.F.$_{10.2}$, in that order, is their gradual decrease in viscosity. Concerning the component previously designated as X_3, indications pointed to a polyvalent alcohol, more specifically glycerol, based on the facts that: it remained in a liquid state and behaved consistently neutral in electrophoresis under different pH conditions, was not susceptible to coloration on the paper chromatogram, and showed no u.v. absorption. Furthermore, the possible presence of a polyhydroxyderivative of cyclohexane, in all probability myo-inositol, had to be considered. The micro-reaction with vanadium oxinate for primary, secondary, and tertiary alcohols[8,9] was positive and showed the same shade of purple as that given by one of the reference substances, glycerol. The presence of a polyhydroxyderivative of cyclohexane was confirmed by means of an extremely sensitive color reaction specific to this class of compounds, a reaction based on the formation of red water-insoluble alkaline earth salts with certain keto acids[10]. In the case of inositol the particular keto acid is leuconic acid, which is the main oxidation product of this alcohol by nitric acid. A further proof of these substances was found in paper chromatography with corresponding reference substances. As solvents we used *n*-butanol–acetic acid–water (4:1:5) and phenol–*o*-cresol (1:1), water-saturated. At 25 ± 2° the R_F values are, in the former solvent, 0.46 for glycerol and 0.07 for myo-inositol; in phenol–*o*-cresol the values are 0.70 and 0.07, respectively. Since in nature there occur two further polyhydroxyderivatives of cyclohexane, mytilyte and scyllite, whose paper chromatographic behavior on a short run is similar to that of myo-inositol, several sheet lengths were run using water-saturated phenol as solvent. Here too, complete identity of the natural product with myo-inositol as the reference substance was found. For displaying the separated substances on the paper chromatogram the method of KARRER *et al.*[11] was used. The thoroughly dried chromatogram was sprayed with a 0.5 % solution of sodium periodate, allowed to react for 5 min at room temperature, then sprayed with a 0.5 % benzidine solution (0.5 g of benzidine in 20 ml acetic acid and 80 ml ethanol). White spots appear on a deep blue background indicating the location of the oxidized substances (Fig. 1).

The carbohydrates contained in the neutral fractions were also affected by this procedure (see Fig. 2). The presence of hexoses in the dialysed axoplasm of both

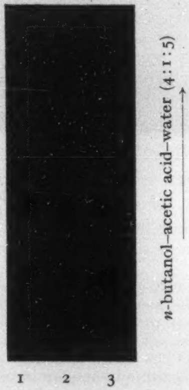
Loligo and *Dosidicus* was indicated by the pure green solution obtained with anthrone[12,13]. However, if the dialyzable portion of the axoplasm is permitted to react with orcine, an olive-green color is obtained. Hexoses which are ketoses react with orcine[14,15] to produce a reddish-brown color, whereas pentoses and pentose derivatives yield a deep green color (Bial's reaction). These reactions are not inhibited by the large excess of other components in axoplasm as shown by the admixture of small known quantities of the carbohydrates in question to the material. Since the reaction of a reference mixture of fructose and ribose with orcine also led to an olive-green solution (mixture of reddish-brown and green), the presence of at least one ketose and one pentose was certain. To test for free sugars, which at all pH values of the buffer system used behave electrically neutral, reactions were carried out with anthrone and orcine on fractions N.F.$_{3.9}$, N.F.$_{2.4}$, and N.F.$_{10.2}$ from both species. With anthrone, green coloring was obtained; with orcine, reddish-brown, which in the neutral fraction clearly indicates the presence of the aldohexose class and the ketohexose class.



1: Glycerol (Reference Subst.)
2: Glucose
3: Fructose
↕
Sucrose
4: L.M.P.$_{D\,g.}$, Neutral Fract. at pH 10.2
5: Inositol
6: L.M.P.$_{L.g.}$, Neutral Fract. at pH 10.2

Fig. 1. The appearance of glycerol, myo-inositol and part of the carbohydrates using the periodate method after paper chromatographic separation from the corresponding neutral fractions. *Loligo*, and sometimes *Dosidicus*, too, leave a stain at the starting point; this stain shows the typical color given by phosphate in reaction with molybdic acid after reduction, and its paper chromatographic behavior under the conditions mentioned also agree with a carbohydrate phosphate. Inferred is the presence of a loose addition complex in order to explain the neutral behavior of this substance during electrophoresis.

The anionic fractions in the region of the glutamic acid ⟷ phosphate fraction were tested for the presence of carbohydrate phosphoric acid esters, using anthrone as reagent. These tests in general turned out negative, while the use of orcine in the region of the glutamic acid fraction produced intensive green coloring after only brief boiling. With the electrophoretic deflection and the curious fact that muscle adenylic acid reacts faster and 1.3 times more strongly with orcine than does free ribose[15], the conclusion was that 5′-AMP must be involved, especially since its presence was indicated theoretically. Of the three reference substances, 5′-AMP, ADP, and ATP, only the first showed the same rate of deflection in electrophoresis as the substance being sought. In contradistinction to yeast adenylic acid, muscle adenylic acid is, by its constitution, capable of giving a positive periodate reaction. Therefore this reaction was carried out on the paper chromatogram, giving the expected positive results; 5′-AMP, ADP and ATP, and yeast adenylic acid were included in the same run as reference substances. U.V. absorption served to determine the location of the spots. The absorption maximum of the glutamic acid fraction, which carried the muscle adenylic acid, was at 260 mμ, which was in exact accordance with the absorption maximum of muscle adenylic acid, used as a reference substance.



1: Fructose ↕ Sucrose
2: L.M.P.$_{L.p.}$, Neutral fract. at pH 10.2
3: L.M.P.$_{D.g.}$, Neutral fract. at pH 10.2

Fig. 2. The paper chromatographic identification of sucrose and fructose with anthrone as coloring reagent.

The identification of individual carbohydrates was achieved by comparing their R_F values with the corresponding reference substances on the paper chromatogram, using various solvent systems. Aliquots of N.F.$_{2.4}$ and N.F.$_{10.2}$ from both species were investigated in this manner, using as spray reagents aniline phthalate[16] for reducing carbohydrates and naphthoresorcine[17,18] for non-reducing carbohydrates. In regard to the colorings with ketoses, those made with anthrone were especially satisfactory[19]. Glucose and fructose were identified without difficulty, and the presence of sucrose was also quickly established (Fig. 2). The proof that it actually was sucrose was established by mild hydrolysis in hydrochloric acid (0.1 *N* HCl, 6 h, 100°); after this hydrolysis this substance could no longer be found; the color of the glucose and fructose spots became more intense, but no additional spot could be observed. The free glucose and fructose found are not products of the hydrolysis of sucrose; the experimental conditions under which the entire separation and processing were carried out precluded such a possibility.

It is probable that considerable proportions of fructose and glucose occur in the form of their phosphoric acid esters in the axoplasm of the living animal. After the death of the animal there takes place a very rapid enzymic cleavage of the phosphoric acid esters. The weak and not always reproducible reactions with anthrone in the region of the glutamic acid—and aspartic acid—fractions, obtained from axoplasm material collected at different times, seem to indicate a scattered presence of such phosphate esters. However, the formation of loose addition complexes must be considered, such as those thought to occur in axoplasm. These present problems in the isolation of pure components.

For the quantitative estimation of glycerol and inositol the method of HIRST[20] was employed, in which the formic acid, obtained by oxidation of those substances with periodate, is determined by titration. First, aliquots of N.F.$_{2.4}$ and N.F.$_{10.2}$ of both species were used, then aliquots of glycerol and inositol which had been separated by paper chromatography using the solvent system *n*-butanol–ethanol–water (4:5:1) and then eluted with water. In the latter case a reference mixture of glycerol and myo-inositol was the basis for calculations; this mixture was separated by paper chromatography under identical conditions. Eluates of substance-free paper strips served as blanks. Since in the former procedure the carbohydrates are also involved, it became necessary to ascertain the accurate number of moles of the liberated formic acid of each participant in the reaction (see ref. 20).

It was necessary to know the individual amounts of carbohydrates, the sum total of which was determined with anthrone by the difference procedure (extinction of maximum absorption at 625 mμ minus extinction of minimum absorption at 700 mμ). To find the concentrations, reference was made to standard solutions of glucose. The individual values were calculated from the size of the spots and the color intensity of the paper-chromatically-separated substances by comparison with the reference substances in various concentrations, run concurrently on the chromatogram. The sum total of the concentrations obtained showed good agreement with the total concentration obtained with anthrone in solution.

Periodate oxidation in solution for determination of glycerol and inositol proceeded as follows: Aliquots of N.F.$_{2.4}$ and N.F.$_{10.2}$ containing 1 to 3 mg polyalcohols (plus carbohydrates), the respective eluates of glycerol and inositol from the paper chromatogram (the substances were applied as a linear streak 30 cm in length), were dissolved in 30 ml of water and treated with 1 ml 0.25 *M* sodium periodate solution. The mixture was boiled for 20 min in the waterbath, cooled, and 0.2 ml ethylene glycol added to destroy the excess periodate. A drop of methylene red was added and the formic acid which had formed was titrated with 0.01 *N* NaOH. As a basis for the quantitative calculation, the results from titrated standard solutions of the two polyalcohols and the carbohydrates respectively were used. Each experiment was accompanied by a blank. The total values obtained from N.F.$_{2.4}$ and N.F.$_{10.2}$ were in good agreement with one another and, after the deduction of the very small values calculated for the carbohydrates, in good agreement with the individual values obtained for glycerol and inositol by the eluating method. The concentrations found for glycerol, inositol and carbohydrates are summarized in Table II.

The quantitative estimation of the 5′-AMP was carried out in solution with orcine on the total axoplasm dialysate as well as on the glutamic acid fraction. Standard solutions of 5′-AMP in various concentrations which were added to the D.P. showed that the reaction was not influenced by any of the components of the dialyzable portion. It was somewhat surprising that only traces could be found of 5′-AMP in the glutamic acid fraction of the dialyzable portion of *Loligo* axoplasm. However, the explanation is simple: an enzymic cleavage, freeing pyrophosphoric acid from ATP, *post mortem*, is to a considerable extent suppressed due to the fact that, for the dissection and preparation of the much smaller *Loligo*, the Institute laboratories afford more favorable conditions. The ATP should be found in the region of the phosphate fraction of *Loligo*, but since there is an insufficient quantity of this fraction, an estimation of ATP could not be made from this material. The concentrations of the substances in *Dosidicus* axoplasm dialysate are given in Table II.

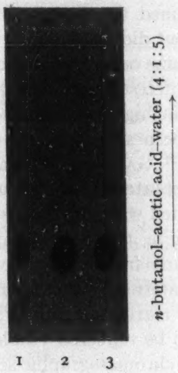
TABLE II

Component	*Loligo*		*Dosidicus*	
	µmoles/100 mg D.P. (dry weight)	*Weight percent*	*µmoles/100 mg D.P. (dry weight)*	*Weight percent*
Glycocoll–betaine $\cdot H_2O$	68.00	9.20	113.00	15.40
Glycerol	81.60	7.50	58.00	5.30
Myo-inositol $\cdot 2H_2O$	7.00	1.50	9.00	1.98
Glucose $\cdot H_2O$	0.22	0.04	0.22	0.04
Fructose	0.22	0.04	0.22	0.04
Sucrose	0.23	0.08	0.23	0.08
Homarine $\cdot H_2O$	18.80	2.92	20.30	3.14
Hypoxanthine	0.70	0.10	0.70	0.10
5′-AMP	—	—	0.45	0.17

Homarine: KOECHLIN[21] has already mentioned this component of squid axoplasm. An aromatic betaine, homarine behaves strictly neutral under every condition of electrophoresis; its absorption maximum lies at 273 mµ; with Dragendorff's reagent[22] it yields the color of a quaternary ammonium compound, though only faintly. The substance was positively identified by paper chromatography. As reference substance the synthetic product was used, according to the directions of HOPPE-SEYLER[23].

In the course of the quantitative evaluation of the components of the N.F.$_{10.2}$ of *Loligo* and *Dosidicus*, we repeated the estimation for homarine and arrived at somewhat higher values than those given in our first paper[1]. Measurements were made with the Beckman spectrophotometer (Model DU) at 273 mµ and 290 mµ of the extinctions of aliquot portions of *Loligo* and *Dosidicus* dialysates and of the two neutral fractions N.F.$_{10.2}$; in parallel we measured an aqueous standard solution of the synthetic substance. The concentrations are given in Table II.

Glycocoll-betaine and hypoxanthine: The test for betaine was made by fusion with metallic potassium which distinctly showed the escape of trimethylamine. The paper chromatogram of the elctrophoretic fraction in question had already shown as main component a quaternary ammonium compound by the positive reaction with Dragendorff's reagent. Since the reaction was delayed, the presence of a carboxyl group was likely. The lack of u.v. absorption pointed to the presence of an aliphatic compound. All these criteria agreed well with the presence of glycocoll-betaine, which gave the same behavior on the paper chromatogram as the substance being sought (Fig. 3). When conditions during electrophoresis were optimal, it was possible to separate betaine from X_2, whereby the latter substance showed a somewhat stronger deflection toward the cathode. For the quantitative determination of these substances we used in each case 200 mg of dialyzed axoplasm from both species of squid, transformed the isolated betaine to its hydrochloride and weighed it. Corresponding concentrations of the betaine, which crystallizes with one molecule of water of crystallization, are shown in Table II.



1: Glycocoll–Betaine·HCl (Ref.-Subst.)
2: L.M.P.$_{\text{D.g.}}$, Fract. II at pH 2.4
3: L.M.P.$_{\text{L.p.}}$, Fract. II at pH 2.4

Fig. 3. Paper chromatographic separation of the betaine- and hypoxanthine fraction after spraying with Dragendorff's reagent. It accords with our earlier investigations showing that the difference in concentration of these new-found components in the D.P. of the axoplasm of both squid species may be quite considerable between otherwise identical molecules. This difference, however, is by and large restricted to the molecules which remain neutral at pH 7.

The u.v. absorbing substance X_2 could not be trigonelline, the isomer of homarine, primarily because trigonelline shows a distinctly different R_F value (0.5) in the butanol–acetic acid system. Neither could it be picolinic acid because, although its R_F value agreed very well with that of picolinic acid in the solvent mentioned, the reaction with Fe^{II} salts was negative, whereas it is characteristically positive in the case of pyridine-2-carbonic acids. Nevertheless, based on the cathodic deflection at pH 2.4, the presence of a basic group had to be assumed. The solution to the problem was finally found in the compound's susceptibility to coloration with eosine on the paper chromatogram; coloration is positive for the purine derivatives adenine, guanine, and hypoxanthine[24]. The pyrimidine derivatives uracil and thymine, which are also u.v.-absorbent, do not respond to this reaction which furnishes purple-red stains and is very sensitive. Proof was found in the identical R_F values of X_2 and the reference substance hypoxanthine ($R_F = 0.42$ in n-butanol–acetic acid). The concentrations of both substances are given in Table II.

The quantitative analysis consisted in calculating the u.v. absorption at the maximum of 251 mμ with reference to a standard solution of synthetic hypoxanthine.

DISCUSSION

Of the free ninhydrin-negative substances discussed here, glycocoll-betaine, glycerol, myo-inositol, hypoxanthine and the carbohydrates glucose, fructose and sucrose had not previously been identified in the dialyzed squid axoplasm. KOECHLIN[21] has already drawn attention to homarine, which was also recognized early in our own analyses. He has also determined the amount of total carbohydrates in the non-dialyzable part of axoplasm, but did not identify the individual carbohydrates.

Taurine and glycocoll-betaine occur in the axoplasm of both species of squid and in high concentrations. It is interesting to note that these two substances, together with glycine, are found also in high concentrations in the muscle tissue of other marine invertebrates, as has been shown by the extensive research of ACKERMANN *et al.*[25–27], and other authors[28–31]. GASTEIGER[32] thinks that the widespread occurrence of homarine in marine invertebrates[25, 33–37], in contradistinction to fresh water invertebrates, is possibly connected with an osmosis-regulating function.

It is interesting that mytilite, the monomethyl derivative of inositol, as well as hypoxanthine, were isolated early from marine invertebrates[25, 26, 38–40].

It is quite possible that quaternary ammonium compounds, such as those already known from extracts of lower marine animals[26, 35, 37], will also be found among the traces of substances which can be rendered visible with Dragendorff's reagent from dialyzed axoplasm after paper chromatographic separation of enriched basic electrophoretic fractions.

No suggestion can be made as to the function of the high concentrations of glycocoll-betaine in the axoplasm. The same must be said of the considerable amounts of free glycerol, which is found only in traces in the brain. The presence of free myo-inositol in the axoplasm is not too surprising. Its presence in free form has been detected in the brain and various other organs[41, 42], in the blood plasma[41–44], and in the thyroid gland of various species[45–47]. Its concentrations in the thyroid are considerable and partly exceed those in the axoplasm of the giant nerve fiber. It has been shown that certain strains of human cells need myo-inositol for their propagation[43, 45–47].

Recently the discovery of hyaluronic acid in the axoplasm and neurilemma of amphibian and mammalian peripheral nerves was announced[48], and we have a recent description of the myo-inositol metabolism in the rat which proceeds by way of D-glucoronic acid[49]. KOECHLIN[21] found glucosamine in the non-dialyzable portion of squid axoplasm, and we confirmed his findings.

The possible role of sucrose in the chemical economy of squid nerve remains to be determined*.

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These investigations are part of a continuing program of biophysical and biochemical research on nerve under the direction of Professor F. O. SCHMITT to whom we wish to express our appreciation for advice and assistance.

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* Since the preparation of this manuscript, our attention has been called to the paper by Y. KOJIMA AND H. KUSAKABE, Isolation of Natural Substances by Ion Exchange Resins, VI[50].

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REFERENCES

[1] G. G. J. Deffner and R. E. Hafter, *Biochim. Biophys. Acta*, 32 (1959) 362.
[2] G. G. J. Deffner and R. E. Hafter, *Biochim. Biophys. Acta*, 35 (1959) 334.
[3] H. B. Steinbach and S. Spiegelman, *J. Cellular Comp. Physiol.*, 22 (1943) 187.
[4] R. D. Keynes and P. R. Lewis, *J. Physiol. (London)*, 114 (1951) 151.
[5] B. L. Vallee, see B. A. Koechlin, *J. Biophys. Biochem. Cytol.*, 1 (1955) 511.
[6] G. G. J. Deffner and R. E. Hafter, *Biochim. Biophys. Acta*, 42 (1960) 200.
[7] W. Grassmann, K. Hannig and M. Ploeckl, *Z. physiol. Chem., Hoppe Seyler's*, 299 (1955) 258.
[8] F. Buscarons, J. L. Marin and J. Claver, *Anal. Chim. Acta*, 3 (1949) 310, 417.
[9] F. Feigl, in *Spot Tests in Organic Analysis*, Elsevier Publishing Co., 1956, p. 172.
[10] F. Feigl and V. Gentil, *Mikrochim. Acta*, (1955) 1004.
[11] M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, 38 (1955) 642.
[12] W. E. Trevelyan and J. S. Harrison, *Biochem. J.*, 50 (1952) 298.
[13] E. W. Yemm and A. J. Willis, *Biochem. J.*, 57 (1954) 508.
[14] W. Mejbaum, *Z. physiol. Chem., Hoppe Seyler's*, 258 (1939) 117.
[15] H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.*, 167 (1947) 368.
[16] S. M. Partridge, *Nature*, 164 (1949) 443.
[17] W. G. C. Forsyth, *Nature*, 161 (1948) 239.
[18] S. M. Partridge, *Biochem. J.*, 42 (1948) 238.
[19] R. Johanson, *Nature*, 172 (1953) 956.
[20] E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, (1949) 1659.
[21] B. A. Koechlin, *J. Biophys. Biochem. Cytol.*, 1 (1955) 511.
[22] H. M. Bregoff, E. Roberts and C. C. Delwiche, *J. Biol. Chem.*, 205 (1953) 565.
[23] F. A. Hoppe-Seyler, *Z. physiol. Chem., Hoppe Seyler's*, 222 (1933) 105.
[24] H. Michl, *Naturwissenschaften*, 40 (1953) 390.
[25] D. Ackermann and R. Janka, *Z. physiol. Chem., Hoppe Seyler's*, 298 (1954) 65.
[26] D. Ackermann and R. Janka, *Z. physiol. Chem., Hoppe Seyler's*, 296 (1954) 283.
[27] D. Ackermann, *Z. physiol. Chem., Hoppe Seyler's*, 232 (1935) 206.
[28] A. C. Kurtz and J. M. Luck, *J. Biol. Chem.*, 111 (1935) 577.
[29] M. Mohr, *Z. Biol.*, 98 (1937) 120.
[30] H. Fuchs, *Z. Biol.*, 98 (1937) 430.
[31] R. H. Chittenden, *Ann. Chem. Pharmaz.*, 178 (1875) 266.
[32] E. L. Gasteiger, J. Gergen and P. Haake, *Biol. Bull.*, 109 (1955) 345.
[33] F. Hanrowitz and H. Waelsch, *Z. physiol. Chem., Hoppe Seyler's*, 161 (1926) 300.
[34] F. A. Hoppe-Seyler, *Z. physiol. Chem., Hoppe Seyler's*, 222 (1933) 105.
[35] D. Ackermann, *Z. physiol. Chem., Hoppe Seyler's*, 295 (1953) 1.
[36] D. Ackermann and P. H. List, *Z. physiol. Chem., Hoppe Seyler's*, 306 (1957) 260.
[37] J. H. Welsh and P. B. Prock, *Biol. Bull.*, 115 (1958) 551.
[38] B. C. P. Jansen, *Z. physiol. Chem., Hoppe Seyler's*, 85 (1913) 231.
[39] D. Ackermann, *Ber.*, 54 (1921) 1938.
[40] D. Ackermann and F. Kutscher, *Z. Untersuch. Nahr. u. Genussm.*, 13 (1907) 180, 610; 14 (1907) 687.
[41] B. S. Platt and G. E. Glock, *Biochem. J.*, 37 (1943) 709.
[42] D. A. Nixon, *J. Physiol. (London)*, 127 (1955) 2.
[43] R. Shihman Chang, *Proc. Soc. Exptl. Biol. Med.*, 99 (1958) 99.
[44] N. Freinkel, R. M. C. Dawson, S. H. Ingbar and R. W. White, *Proc. Soc. Exptl. Biol. Med.*, 100 (1959) 549.
[45] H. Eagle, *Science*, 122 (1955) 501.
[46] H. Eagle, V. I. Oyama and M. Levy, *Science*, 123 (1956) 845.
[47] H. Eagle, V. I. Oyama, M. Levy and A. E. Freeman, *J. Biol. Chem.*, 226 (1957) 191.
[48] L. G. Abood and S. K. Abul-Haj, *J. Neurochem.*, 1 (1956) 119.
[49] J. J. Burns, N. Trousof, C. Evans, N. Papadopoulos and B. W. Agranoff, *Biochim. Biophys. Acta*, 33 (1959) 215.
[50] Y. Kojima and H. Kusakabe, *J. Sci. Res. Inst. Tokyo*, 51 (1957) 189.

CHEMICAL INVESTIGATIONS OF THE GIANT NERVE FIBERS OF THE SQUID

IV. ACID–BASE BALANCE IN AXOPLASM

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SUMMARY

Acid–base balance is substantially established for the dialyzable portion of the axoplasm of the giant nerve fiber of *Loligo pealii* and *Dosidicus gigas*. All the free components of this axoplasm which have so far been identified and quantitatively estimated are listed. For *Loligo pealii* this total portion is 93 %, for *Dosidicus gigas* 95 % of the weight of solutes present.

INTRODUCTION

With the discovery of large amounts of free dicarboxylic amino acids[1–6] and of isethionic acid[5] in squid axoplasm the identity of the large amounts of organic anions needed to balance the inorganic cations seemed to have been substantially determined. KOECHLIN's analyses suggested a cation deficit amounting to some 80 μequiv. (out of 520 μequiv.) and suggested that this may be composed of unidentified organic bases.

The analysis in these investigations of the dialyzable portion of squid axoplasm has identified about 95 % of its constituents. The only organic cations found were arginine, lysine and ornithine in relatively low concentration. The presence of organic bases, such as quaternary ammonium compounds, in amounts totaling 80 μequiv./g was thereby excluded. No evidence has been obtained for other organic acids, such as "axonic" acid, "sulfonate x", fumaric and succinic acid, thought by KOECHLIN to occur in the peripheral nerves of the lobster, crab and rat, as well as in squid.

Because of the key role of isethionic acid in the acid–base balance in squid axoplasm an independent determination of its concentration was made for comparison with KOECHLIN's value. The result was substantially lower: 19 % as compared with the 28 % found by KOECHLIN. This reduced correspondingly the necessity for organic base whose existence seemed to have been excluded by analyses already completed. In these estimates we assumed the values of inorganic base as determined in *Loligo* axoplasm independently by these groups of investigators[9–11].

EXPERIMENTAL

Material and methods

The dialyzed axoplasm used in the investigations came from the same sample used in all previously reported analyses[6–8]. The methods of electrophoretic fractionation were given in detail in earlier papers[6–8].

If, at pH 3.9 in an acetic acid–pyridine buffer, one feeds the dialyzed axoplasm not at the center, but near the cathode of the paper curtain, thereby sacrificing the collection of the basic components, one obtains under optimal conditions in descending order of their migration rates: chloride, sulfate, isethionic acid and phosphate as pure fractions. The quantitative collection of each separate fraction is facilitated if, after termination of the separation, a control experiment is carried out under identical conditions and the result observed under u.v. light.

Estimation of isethionic acid

Direct weighing of the fractions, even when thoroughly dried, is not practicable, because the strong acid retains tenaciously some pyridine from the buffer. Moreover, the free acid is syrupy and strongly hygroscopic. The quantitative estimation was gravimetric and titrimetric, with control experiments run concurrently. The gravimetric method consisted of oxidation with nitric acid in the sealed tube and precipitation of the resulting sulfate in the form of barium sulfate[12]. It was investigated with the pure fractions as well as with the total dialyzable portion; in the former case 20–40 mg were used, in the latter approx. 100 mg. The relatively small amounts of *Loligo* material available allowed only one estimation each. This procedure also involved the sulfur-containing components already quantitatively determined with ninhydrin, *i.e.*, taurine, cysteic acid amide, methionine, and methionine-sulfoxide, the latter recently found by us. Consequently, we were able to determine, after deduction for the values of the above substances, whether there were any sulfur-containing substances in substantial concentrations present in free form in the axoplasm. The results were unequivocally negative.

In the titrimetric method, the pyridine was first expelled by adding 10 ml of 0.01 *N* NaOH to aliquots of isethionic acid (approximately 5 mg in each case) and boiling for 5 min; the odor of the escaping pyridine was distinctly perceptible. Subsequently the added NaOH was neutralized with 0.01 *N* HCl, then the sulfonate titrated with 0.01 *N* NaOH, using phenolphtaleine as indicator. The results given in Table I represent the mean values obtained from the gravimetric and titrimetric estimations.

Estimation of chloride, sulfate, and phosphate

Chloride and sulfate were estimated gravimetrically as AgCl and $BaSO_4$, respectively. Phosphate was determined from the fraction and from the total dialysate by spectrophotometry at 830 mμ following the somewhat modified method of BOLTZ AND MELLON[13]. The fractions, individually collected after the electrophoretic separation, were freed of buffer in the desiccator, an excess of 0.1 *N* NaOH having been added previously to the chloride fraction.

Estimation of potassium

For this the directions of PIPER[14] were followed. The method is based on the precipitation of the potassium in the form of the practically insoluble potassium-sodium-cobalt^{III}-nitrite and subsequent oxidation of the nitrite into nitrate by means of permanganate. Three reference tests conducted with potassium chloride led in each case to values which were somewhat too high, with an average error of about 3 %. The inorganic cations of aliquots of dialyzed *Dosidicus* axoplasm (approx.

TABLE I

ACID-BASE BALANCE IN SQUID NERVE AXOPLASM (pH 7.0)

Neutral constituents

	Loligo			*Dosidicus*		
	µmoles/ 100 mg D.P.	*Weight percent*	*N in percent of total N*	*µmoles/ 100 mg D.P.*	*Weight percent*	*N in percent of total N*
Glycine	10.70	0.80	3.18	10.40	0.78	3.27
Alanine	7.80	0.79	2.55	9.30	0.83	2.83
Serine	3.70	0.39	1.06	1.00	0.11	0.32
Leucine + Isoleucine	2.70	0.35	0.85	0.20	0.03	0.06
Valine	2.22	0.26	0.64	0.50	0.06	0.15
Threonine	1.90	0.23	0.64	0.30	0.04	0.09
Proline	1.00	0.12	0.42	0.10	0.01	0.04
Tyrosine	0.70	0.13	0.21	0.30	0.06	0.08
Phenylalanine	0.60	0.10	0.19	0.15	0.03	0.04
Methionine	0.50	0.08	0.16	0.30	0.04	0.08
Methioninesulfoxide**	0.30	0.05	0.09	0.20	0.03	0.07
Citrulline**	0.40	0.07	0.36	0.15	0.03	0.16
α-NH_2-butyric acid				0.05	0.01	0.02
Cysteic acid amide	4.50	0.74	2.65	0.04	0.07	0.25
Taurine	98.50	12.33	29.32	31.40	3.93	9.60
Glycocoll-betaine·H_2O	68.00	9.20	20.20	113.00	15.40	34.75
Homarine·H_2O	18.80	2.92	5.55	20.30	3.14	6.05
Glycerol	81.60	7.50		58.00	5.30	
Myo-inositol·$2H_2O$	7.00	1.50		9.00	1.98	
Glucose·H_2O	0.22	0.04		0.22	0.04	
Fructose	0.22	0.04		0.22	0.04	
Sucrose	0.23	0.08		0.23	0.08	
Hypoxanthine	0.70	0.10	0.87	0.70	0.10	0.89
Total neutral	311.99	37.82	68.94	256.42	32.04	58.75

Anions

	Loligo				*Dosidicus*			
	µequiv./ 100 mg D.P.	*µmoles/ 100 mg D.P.*	*Weight percent*	*N in percent of total N*	*µequiv./ 100 mg D.P.*	*µmoles/ 100 mg D.P.*	*Weight percent*	*N in percent of total N*
Bicarbonate*								
Chloride	140.00	140.00	4.96		142.00	142.00	5.02	
Sulfate					14.20	7.10	0.68	
Phosphate HPO_4''– H_2PO_4' 61.4/38.6	26.60	16.50	1.57		22.75	14.10	1.34	
5'-AMP					0.90	0.45	0.17	0.72
Isethionic acid	152.00	152.00	19.10		157.00	157.00	19.80	
Aspartic acid	73.00	73.00	9.72	21.65	105.60***	105.60	14.05	31.42
Glutamic acid	19.60	19.60	2.88	5.81	26.20	26.20	3.85	8.06
Total acid	411.20	401.10	38.23	27.46	468.65	452.45	44.91	40.20

* Not determined.

** Methioninesulfoxide and Citrulline were given in an earlier paper the tentative designation N_2 and N_1, respectively[6].

*** Value given in the first paper was found to be too low[6].

Continued TABLE I

	Cations							
	Loligo				*Dosidicus*			
	μequiv./ 100 mg D.P.	*μmoles/ 100 mg D.P.*	*Weight percent*	*N in percent of total N*	*μequiv./ 100 mg D.P.*	*μmoles/ 100 mg D.P.*	*Weight percent*	*N in percent of total N*
Potassium	344.00	344.00	13.40		388.00	388.00	15.18	
Sodium	65.00	65.00	1.50		77.00	77.00	1.77	
Calcium	7.00	3.50	0.14		7.00	3.50	0.14	
Magnesium	20.00	10.00	0.24		20.00	10.00	0.24	
Arginine	3.20	3.20	0.56	4.24	4.10	4.10	0.71	5.88
Lysine	2.40	2.40	0.35	1.48	0.20	0.20	0.03	0.13
Ornithine	1.80	1.80	0.24	1.06	0.30	0.30	0.04	0.17
Total base	443.40	429.90	16.43	6.78	496.60	483.10	18.11	6.18
			Weight percent	*N in percent of total N*			*Weight percent*	*N in percent of total N*
TOTAL			92.48	103.18			95.06	105.13

50 mg, dried to constant weight) were converted to sulfates by means of concentrated sulfuric acid and concentrated nitric acid, the organic components being destroyed. The sulfates were then weighed. The small concentration of the phosphate in the dialysate interfered very little, as phosphate and sulfate have almost the same molecular weight. The potassium, precipitated from its sulfate with potassium-sodium-cobalt^{III}-nitrite as complex salt, was filtered off and washed in accordance with the conditions of the gravimetric analysis. The precipitate was dissolved with a measured excess of a sulphuric acid–permanganate solution under heat, and after addition of a known quantity of oxalic acid retitrated with permanganate.

The values for calcium and magnesium found by VALLEE[11] for *Loligo* axoplasm were used in the acid–base estimate for *Dosidicus* axoplasm. In view of the very low concentrations of these cations this procedure seemed justified. The value given by us for sodium was estimated from the difference and is therefore less reliable.

Tests for polycarboxylic acids and oxo-carboxylic acids

Through preliminary tests, information was obtained about the electrophoretic deflection at pH 3.9 and the paper chromatographic behavior of those acids which are members of the citric acid cycle. These substances were made visible on paper and determined in solution[15,16]. The information thus gained was applied to the appropriate fractions of the axoplasm of both species of squid. No acid belonging to this class of compounds was found despite KOECHLIN's statement that succinic and fumaric acids occur in *Loligo* axoplasm. To eliminate all doubt the following procedure was adopted: we subjected to further electrophoretic separation at pH 2.4 those anionic fractions which since the beginning of our investigations of *Dosidicus* axoplasm have become enriched through numerous electrophoretic separations at pH 3.9 and within whose area the two acids mentioned ought to be found. At pH 2.4 the acid amino acids are weakly deflected toward the cathode, whereas the polybasic acids run neutral. However, the paper chromatographic examination of this neutral fraction (collected on a broader base) with MUNIER's method[15], which allows sharp separations, did not yield any results, nor did the Baeyer test which is sensitive for olefinic double bonds.

ANALYTICAL RESULTS

The correction of the value for isethionic acid, the fact that we were unable to find the two polycarboxylic acids mentioned by KOECHLIN, and the fact that we see no indication of the presence of further acids, lead to the result, contrary to KOECHLIN's investigations, that there is now a deficit of approximately 30 μequiv. on the anion side of the acid–base balance of the squid axoplasm.

Theoretically, the balance might be established on the basis of the following considerations: for the brain, plasma, and cerebrospinal fluid of man, HARRISON[17] and MANERY[18] have found bicarbonate concentrations of 12, 27, and 18 equiv./g or ml, respectively. Since all these values are based on wet weight, their conversion to dry weight would increase the anion side of the acid–base balance of the axoplasm on the average, by about 15 μequiv. Furthermore, if we put into the acid–base balance of the axoplasm of *Loligo* the sulfate concentration, which in *Dosidicus* amounted to 15 μequiv., we would then find the balance re-established. The small excess of cations in the balance which would follow from this explanation might possibly be attributed to a slight overestimation of the potassium value.

In Table I are shown all free components of the dialyzable part of the axoplasm found to date. The portion of the *Loligo* axoplasm identified at the present date amounts to 93 %, that for *Dosidicus* to 95 %. But it is to be assumed that the proportion of the known substances in the dialyzed axoplasm of both species is actually somewhat higher, since experience has shown that the dialyzable portion of the axoplasm even after thorough drying in the desiccator, still tenaciously retains water which can be temporarily freed in the drying oven. This retention of water is primarily due to the isethionic acid, already mentioned. In the investigation of such complex material a somewhat larger margin of error must be expected in the quantitative estimations.

Since the theoretically possible value for total nitrogen is already exceeded by a few percent, additional nitrogenous compounds (for the presence of which we have proof) can be expected to be found only in small concentrations.

DISCUSSION

From analyses of the nerves of several invertebrate types (see ref. 19), aspartic acid is found to be prominent in all. Its function in nerve is probably not restricted to the maintenance of the ion balance alone. Possibly significant in this connection is the fact that the fibrous protein of the axon[20], which has been under study in these laboratories for some time, shows a high total concentration of mono-amino-dicarboxylic acids, with glutamic acid as the principal component, and also a high percentage of amide nitrogen[21].

Since there is no appreciable concentration of organic base in the dialyzable portion of squid axoplasm, such substances would appear to be excluded as carriers of the bioelectric stimulating current.

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These investigations are part of a continuing program of biophysical and biochemical research on nerve under the direction of Professor F. O. SCHMITT to whom we wish to express our appreciation for advice and assistance.

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REFERENCES

[1] R. S. BEAR AND F. O. SCHMITT, *J. Cellular Comp. Physiol.*, 14 (1939) 205.
[2] F. O. SCHMITT, R. S. BEAR AND R. H. SILBER, *J. Cellular Comp. Physiol.*, 14 (1939) 351.
[3] R. H. SILBER AND F. O. SCHMITT, *J. Cellular Comp. Physiol.*, 16 (1940) 247.
[4] R. H. SILBER, *J. Cellular Comp. Physiol.*, 18 (1941) 21.
[5] B. A. KOECHLIN, *J. Biophys. Biochem. Cytol.*, 1 (1955) 511.
[6] G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 32 (1959) 362.
[7] G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 35 (1959) 334.
[8] G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 42 (1960) 189.
[9] H. B. STEINBACH AND S. SPIEGELMAN, *J. Cellular Comp. Physiol.*, 22 (1943) 187.
[10] R. D. KEYNES AND P. R. LEWIS, *J. Physiol.*, (*London*). 114 (1951) 151.
[11] B. L. VALLEE, see B. A. KOECHLIN, *J. Biophys. Biochem. Cytol.*, 1 (1955) 511.
[12] O. HOENIGSCHMID AND E. ZINTL, in *Anleitung zur quantitativen Gewichtsanalyse*, München, 1921, Chem. Lab. d. Staates.
[13] D. F. BOLTZ AND M. G. MELLON, *Anal. Chem.*, 19 (1947) 873.
[14] C. S. PIPER, *Handbuch der Analytischen Chemie*, 3. Teil, Band Ia, Berlin, Springer Verlag, 1940.
[15] R. MUNIER, *Bull. soc. chim. France*, (1952) 852.
[16] Z. DISCHE, R. WEIL AND E. LANDSBERG, *J. Biol. Chem.*, 208 (1954) 23.
[17] G. A. HARRISON, in *Chemical Methods in Clinical Medicine*, 3rd Ed., London, Churchill, 1947.
[18] I. F. MANERY, in *The Biology of Mental Health and Disease*, London, Casell, 1952.
[19] F. O. SCHMITT AND N. GESCHWIND, *Progr. in Biophys.*, 8 (1957) 165.
[20] F. O. SCHMITT, *J. Cellular Comp. Physiol.*, 49 (1957) 165.
[21] P. F. DAVISON AND E. W. TAYLOR, *J. Gen. Physiol.*, 43 (1960) 801.

AMINO ACID INCORPORATION INTO PROTEIN BY EXTRACTS OF *E. COLI*

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SUMMARY

The incorporation of amino acids into protein in extracts of *E. coli* is described under conditions which show a dependence on the addition of ATP and an ATP generating system, GTP, Mg^{++}, KCl, and an amino acid mixture. The site of incorporation is the bacterial ribonucleoprotein particle. The addition of the non-sedimentable $105,000 \times g$ supernatant solution stimulates this incorporation. Large bacterial fragments are not required for the incorporation. Ribonuclease and chloramphenicol inhibit it.

In order to permit meaningful interpretation of the cell-free capacity of the system, it has been found essential that the whole cell contamination of these preparations be no greater than $1 \cdot 10^5$ viable cells per ml at the start of a 15-min incubation period. The importance of determining the degree of contamination of bacterial extracts by whole cells in studies on the proteosynthetic mechanism is emphasized.

INTRODUCTION

Studies on the details of amino acid incorporation into protein have led to the postulation of the following sequence of reactions: (a) amino acid activation; (b) transfer of the amino acid to a low molecular weight "soluble" RNA; (c) amino acid incorporation into the protein of the ribosome (ribonucleoprotein particle). Reactions (a) and (b) have been reported in both mammalian and bacterial cell-free systems[1-4]. In addition, Tissières and Watson[5] have shown that the ribosomes of *E. coli* have many physico-chemical properties in common with ribosomes of rat liver described by Petermann *et al.*[6]. These similarities suggested that amino acid incorporation of the type described, using cell-free fractions of rat liver[7,8] might also occur in bacteria. There are a number of reports of bacterial amino acid incorporation systems, *cf.* Simkin[9]. This communication describes the conditions required for amino acid incorporation into extracts of *E. coli*. The results show that the components of this system are similar to those of mammalian systems.

Abbreviations used: RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PEP, phosphoenol pyruvate; PEP kinase, pyruvate kinase (obtained from C. F. Boehringer und Sohne, Mannheim, Germany); CTP, cytosine triphosphate; and UTP, uridine triphosphate. All nucleotides were obtained from the Sigma Company.

MATERIALS AND METHODS

E. coli (K-12), kindly supplied by Dr. L. GORINI, was grown with moderate aeration at 37° on either the minimal medium of DAVIS AND MINGIOLI[10] (omitting the citrate) or on a complete medium which contained peptone. The bacteria were harvested shortly after the population had entered the log phase. The yield varied between 1 and 4 g wet wt./15 l of medium.

Bacterial extracts were prepared by grinding washed whole cells with Alumina (Alcoa A-301, 325 mesh). The alumina and cells (3:1) were homogeneously mixed in an ice cold mortar. To the white, paste-like material a volume of extracting solvent was added equal to the wet weight of cells. The solvent contained: Tris buffer (0.1 *M*, pH 7.9); $MgCl_2$ ($7 \cdot 10^{-3}$ *M*); and KCl ($6 \cdot 10^{-2}$ *M*). The mixture of solvent, cells and alumina was vigorously ground for 5 min. Precipitable material was removed by centrifugation (10 min, 10,000 × *g*). The precipitate was re-extracted two more times as before, except that 2 volumes of extracting solvent were used each time; the supernatants of all centrifugations were then combined and used immediately.

L-[1-^{14}C]leucine was synthesized[11] and kindly furnished by Dr. Robert B. Loftfield. The leucine was diluted to a specific activity of $1.8 \cdot 10^6$ counts/min/μmole. The precipitated protein samples were counted on a Nuclear gas flow counter with a Micromil end window. The counting efficiency was approx. 30%. The washing, plating, and counting procedures described by SIEKEVITZ[12] were used. Leucine incorporation described in this paper was stable to hot trichloroacetic acid extraction.

To determine if the incorporated leucine was fixed in α-peptide linkage, [^{14}C]protein was hydrolyzed by a modification of the method described by ZAMECNIK AND KELLER[7]. Following hydrolysis the protein was dried, dissolved in a minimal amount of water and chromatographed on paper in a solvent consisting of *n*-butanol–water–acetic acid (5:4:1). Ninhydrin positive material and areas of radioactivity (determined by a Nuclear-Chicago model C-100-A Actigraph II) were compared at intervals during the course of hydrolysis, Partial hydrolysis (2 *N* HCl, 3 h, 115°) revealed that the radioactivity had been distributed among four discrete, ninhydrin positive areas. The R_F's were: 0.85, 0.62, 0.36, 0.00. The R_F of free leucine was 0.60, and unhydrolyzed protein was stationary in this solvent. The release of free radioactive leucine increased with the time of hydrolysis. Complete hydrolysis (6 *N* HCl, 24 h, 115°) showed that radioactivity could be located only in the area of free leucine. These results are consistent with the interpretation that, following partial hydrolysis, radioactive leucine is present in peptides which are further degraded to release free leucine on complete acid hydrolysis.

EXPERIMENTAL

Short-term incorporation studies (15 min) using whole cells showed that tracer amounts of leucine are rapidly incorporated into protein without exogenous additions. When cultures of *E. coli* (K-12) are serially diluted and each dilution is incubated with the same tracer amount of [^{14}C]leucine, viable cells are still present at the point where leucine incorporation is insignificant. Fig. 1 shows that, when the number of viable cells is $1 \cdot 10^5$ per ml or less, the amount of labeled protein is not above background. In four experiments conducted in this manner, the range of the inflection point was from $5 \cdot 10^4$ to $1 \cdot 10^6$, with an average value of $3 \cdot 10^5$ viable cells per ml. Extracts prepared as

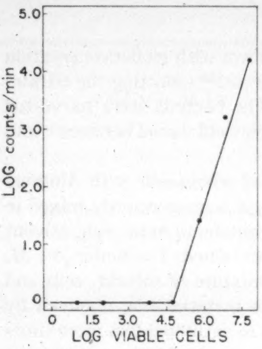

Fig. 1. Whole cell incorporation of L-[1-^{14}C]leucine. A suspension of whole, viable cells ($1 \cdot 10^8$ per ml) was serially diluted in 10-fold steps. 0.2 ml of each dilution was incubated at 37° for 15 min. In addition to the whole cell suspension the complete system contained: Tris ($1 \cdot 10^{-2}$ M, pH 7.8), L-[1-^{14}C]leucine (0.1 μmole, containing $1.8 \cdot 10^5$ counts/min), $MgCl_2$ (7 μmoles), KCl (60 μmoles), final volume 1 ml. Viable cell counts made by the pour plate method (in triplicate). After incubation, 4 mg of carrier protein were added to each flask. Samples which had a count rate between 1 and 0 were plotted as 1.

TABLE I

REQUIREMENTS FOR AMINO ACID INCORPORATION

The complete system contained: $MgCl_2$, $7 \cdot 10^{-3}$ M; KCl, $6 \cdot 10^{-2}$ M; ATP, 1 μmole; GTP, 0.25 μmoles; PEP, 5 μmoles; PEP kinase, 50 μg protein; 18 L-amino acids, 0.2 μmoles each; the mixture of ROBERTS *et al.*[20] minus diaminopimelic acid was used; Tris, $1 \cdot 10^{-2}$ M, pH 7.9; L-[1-^{14}C]leucine, 0.1 μmole, $1.8 \cdot 10^5$ counts/min; 4 mg of crude 10,000 × *g* supernatant of *E. coli* extract. 15 min incubation at 37° with occasional shaking. Final volume 1.0 ml, final pH 7.8.

Incorporation system	*Number of experiments*	*Percentage of "complete" specific activity**
Complete		100*
— Mg^{++}	3	0**
— KCl	6	41 ± 14
— ATP	4	64 ± 17
— GTP	4	76 ± 4
— PEP, PEP kinase	4	47 ± 2
— ATP, GTP, PEP, PEP kinase	3	0.2 ± 0.3
— Amino acids	4	38–80**
+ Ribonuclease (0.01 μg/ml)	1	40
+ Ribonuclease (1.0 μg/ml)	3	4 ± 0.6
+ Chloramphenicol (30 μg/ml)	3	31
+ Chloramphenicol (100 μg/ml)	2	8

* The average specific activity for the "complete" system was 480 counts/min/mg of protein ($2.6 \cdot 10^{-4}$ μM of [1-^{14}C]leucine incorporated/mg of protein). The standard deviation indicates the variation of effect observed in each of the experiments. It is not the variation of duplicate samples.

** See text for details.

described above rarely contain more than $1 \cdot 10^5$ viable whole cells per ml. ROGERS AND NOVELLI[13] report that the efficiency of ornithine transcarbamylase synthesis in protoplasts is increased 7-fold by the addition of bacterial extracts. In contrast to their protoplast preparation, the efficiency of amino acid incorporation into whole cells is not increased by the presence of the cellular milieu during short time incubations.

Incorporation studies in extracts which do not contain a significant number of whole cell contaminants show one striking difference from whole cell incorporation studies. For maximal incorporation, certain additions are required, as shown in Table I. The absence of magnesium completely inhibits the incorporation. The cells are routinely ground, therefore, with alumina in the presence of magnesium. The crude bacterial extract shows the same requirement as liver homogenates for ATP, GTP and the energy generating system[7,8]. Removal of any of these additives reduces the incorporation. If all of these components are withheld, the incorporation level is close to zero. There is no stimulation of incorporation by addition of CTP, UTP, or the four common deoxyribonucleotides.

A mixture of 18 L-amino acids stimulates the incorporation of leucine into protein. No stimulation of leucine incorporation is obtained when only valine and isoleucine are substituted for the 18 amino acid mixture. The stimulation by amino acids is variable and is related to the composition of the medium used for the growth of the intact cells prior to rupture. Maximal stimulation (50–80 %) is observed using extracts of bacteria grown on the complete medium. Extracts of cells grown on minimal medium are stimulated by amino acids (20–40 %) only after a preliminary incubation, presumably to deplete the extract of residual free amino acids.

Low concentrations of ribonuclease (0.01 μg/ml) (Worthington) significantly inhibit the incorporation. Neither *E. coli* soluble RNA (a gift of Dr. A. TISSIÈRES) nor yeast soluble RNA were capable of reactivating the inhibited system. Chloramphenicol (30 μg/ml) also causes marked inhibition of leucine incorporation in the bacterial extract system. This concentration of chloramphenicol also inhibits protein synthesis of whole cells.

TABLE II

INCORPORATING ABILITY OF VARIOUS CELL FRACTIONS

Each of the fractions was incubated for 15 min at 37° in the complete system described in Table I. Specific activity is defined as counts/min/mg of protein, corrected for self absorption. In the case of Fraction 1, this would be equivalent to $4.09 \cdot 10^{-4}$ μM of leucine/mg of protein.

Fraction No.	*Fraction description*	*Total counts/min*	*Specific activity*
1	10,000 × *g* (10 min) supernatant	2870	757
2	30,000 × *g* (20 min) pellet*	0	0
3	30,000 × *g* (20 min) supernatant	2795	777
4	100,000 × *g* (2 h) pellet*	714	340
5	100,000 × *g* (2 h) supernatant	3	3
6	Fractions 2 + 5 (1:1)	14	6
7	Fractions 4 + 5 (1:1)	1734	468
8	Fractions 4 + 5 (1:2)	3000	527
9	Fractions 4 + 5 (1:3)	3419	444

* Pellets were resuspended in Tris buffer (0.01 *M*, pH 7.8) and diluted to the volume of the original 10,000 × *g* supernatant solution.

Utilizing the optimal conditions described in Table I, the amino acid incorporation system can be fractioned by centrifugation as shown in Table II. After high speed centrifugation, there is no incorporation of amino acid by the supernatant fraction alone. When this supernatant solution is added to the fraction sedimenting at 100,000 × *g* (2 h), roughly 70 % of the crude extract incorporation is recovered.

DISCUSSION

These results point out the desirability of assessing the degree of whole cell contamination in each experiment in which incorporation of amino acids into protein in extracts of bacterial systems is being studied. It is necessary in the present system, where a 15-min incubation is employed, to reduce the whole cell contamination to a level of $1 \cdot 10^5$ cell/ml or less, in order to obtain meaningful incorporation values for the cell-free fractions. The risks of whole cell artifact are increased in the presence of an incorporation medium rich in amino acids and nucleotide cofactors, and become increasingly grave with longer incubation times. As shown, contamination can be determined by cell count or by comparing the incorporation of paired flasks in the presence and absence of ATP, GTP, PEP and PEP kinase, since only the incorporation into bacterial extracts requires these additions.

The growth phase of the cells is also important. For maximal incorporation, the cells must be in the earliest stage of log phase growth. Experimentally it has been observed that yields in excess of 4 g of cells (wet wt./16 l of media) give extracts which have specific incorporating activities less than $6 \cdot 10^{-5}$ μmole of leucine/mg of protein.

The present system closely resembles the rat liver cell-free system previously described[7,8,14], in that both require sedimentable and non-sedimentable fractions for maximal incorporation, both are stimulated by ATP, GTP, and a nucleoside triphosphate generating system, and optimal concentrations of each of these additives are comparable. In addition, the kinetics of incorporation are similar (rapid initial incorporation followed by a plateau-ing after 15-30 min incubation). Recently TISSIÈRES AND WATSON found that the magnesium concentration is a critical factor for the stability of the bacterial ribosomes[5], an observation parallel to the findings of PETERMANN *et al.*[6] in animal systems. As shown here, maximal incorporation occurs at the magnesium concentration optimal for stabilizing the 70-S particles described by TISSIÈRES AND WATSON.

The incorporation described herein differs from the *in vitro* bacterial systems of BELJANSKI AND OCHOA[15], SPIEGELMAN[16], GALE AND FOLKES[17] and HUNTER *et al.*[18] in that a low speed precipitable membrane fraction is not required. An additional distinction from the mucopeptide synthesis of MANDELSTAM AND ROGERS[19] is that the present system is chloramphenicol-sensitive.

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REFERENCES

[1] M. B. HOAGLAND, *Proc. 4th Int. Congr. Biochem.*, Vienna, 1958.
[2] *Symposium on Amino Acid Activation, Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 67.
[3] J. A. DEMOSS AND G. D. NOVELLI, *Biochim. Biophys. Acta*, 18 (1955) 592.
[4] B. NISMANN, F. H. BERGMANN AND P. BERG, *Biochim. Biophys. Acta*, 26 (1957) 639.
[5] A. TISSIÈRES AND J. D. WATSON, *Nature*, 182 (1958) 778.
[6] M. L. PETERMANN, M. G. HAMILTON, M. E. BALIS, K. SAMMARTH AND P. PECORA, in R. B. ROBERTS, *Microsomal Particles and Protein Synthesis*, Pergamon Press, London, 1958, p. 70.

[7] P. C. ZAMECNIK AND E. B. KELLER, *J. Biol. Chem.*, 209 (1954) 337.
[8] E. B. KELLER AND P. C. ZAMECNIK, *J. Biol. Chem.*, 221 (1956) 45.
[9] J. L. SIMKIN, in J. M. LUCK, *Ann. Rev. Biochem.*, 1959, p. 145.
[10] B. D. DAVIS AND E. S. MINGIOLI, *J. Bacteriol.*, 60 (1950) 17.
[11] R. B. LOFTFIELD, *Nucleonics*, 1 (1947) 54.
[12] P. SIEKEVITZ, *J. Biol. Chem.*, 195 (1952) 549.
[13] P. ROGERS AND G. D. NOVELLI, *Federation Proc.*, 18 (1959) 1232.
[14] H. SACHS, *J. Biol. Chem.*, 228 (1957) 23.
[15] M. BELJANSKI AND S. OCHOA, *Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 494.
[16] S. SPIEGELMAN, in G. TUMEVALL, *Recent Progress in Microbiology*, Almquist and Wiksell, Stockholm, 1959, p. 81.
[17] E. F. GALE AND J. P. FOLKES, *Biochem. J.*, 59 (1955) 661, 675.
[18] G. D. HUNTER, P. BROOKE, A. R. CRATHORN AND J. A. V. BUTLER, *Biochem. J.*, 73 (1959) 369.
[19] J. MANDELSTAM AND H. J. ROGERS, *Biochem. J.*, 72 (1959) 654.
[20] R. E. ROBERTS, P. H. ABELSON, D. B. COWIE, H. T. BOLTON AND R. J. BRITTEN, *Studies of Biosynthesis* in *E. coli*, Carnegie Institute of Washington, 1955, p. 28.

FURTHER STUDIES ON CYSTATHIONINE SYNTHETASE-SERINE DEAMINASE OF RAT LIVER

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SUMMARY

1. The purity of rat liver cystathionine synthetase-serine deaminase was more than doubled over that previously reported through the use of zone electrophoresis. The ratio of the two enzymic activities of the more purified preparation remained unchanged.

2. Enzymic activity was slightly inhibited by certain pyridoxine derivatives and strongly inhibited by carbonyl reagents, sulfhydryl reagents, various metal chelating agents, and by a number of heavy metal ions. Mg^{++} and Mn^{++} slightly stimulated the serine deaminase activity.

3. Study of the substrate specificity of the enzyme showed that only L-serine and L-threonine were deaminated and only homocysteine was utilized for synthesis of a sulfur-containing diamino acid with L-serine. L-cysteine strongly inhibited cystathionine synthesis.

INTRODUCTION

A procedure for the partial purification of cystathionine synthetase-serine deaminase from rat liver and some of the properties of this enzyme were reported earlier[1]. The present communication deals with the further purification of the enzyme by column electrophoresis, studies on the substrate specificity of its dual enzyme activities, and inhibition and activation by a variety of reagents..

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* On leave of absence from the Abbassia Faculty of Medicine, Abbassia, Cairo, Egypt, to June 1959.

EXPERIMENTAL MATERIALS AND METHODS

Materials

Geon (type 426) resin for use in column electrophoresis was generously supplied by the B. F. Goodrich Chemical Co., Avon Lake, Ohio. It was washed before use and the column was packed in the manner described by PORATH[2].

The chemical compounds employed in this work and their sources are the same as previously reported[1].

Enzyme preparation

Preliminary purification of the enzyme for column electrophoresis was carried out by the published method[1]. This preparation was stored with ammonium sulfate as a frozen thick paste with almost no loss of activity. The serine deaminase specific activity of the preparations varied between 150 and 160.

Analytical methods

Protein concentrations were determined by the method of MOKRASCH AND MCGILVERY[3], based on the biuret reaction.

The enzyme assay methods previously reported[1] were employed, with the exception that mercaptoethanol was substituted for dimercaptopropanol because the former is more stable and more soluble in water, and, also, because at the concentration employed ($1.3 \cdot 10^{-4}$ *M*) mercaptoethanol slightly activated while the dimercaptopropanol somewhat inhibited the serine deaminase reaction.

In the assays, 0.25 mmoles of DL-homocysteine or 0.2 mmoles of L-serine/3 ml of incubation medium was employed as the substrates. The reaction medium contained between 0.35 to 0.45 mg of enzyme protein; 0.1 *M* potassium borate buffer, pH 8.3, and $5 \cdot 10^{-5}$ *M* pyridoxal phosphate with other components as indicated in the respective tables. The incubations were run for 30 min at 37° in a Dubnoff shaking incubator.

For column electrophoresis, the packed column (2 × 55 cm) was washed and equilibrated with 0.05 *M* potassium phosphate buffer, pH 7.5 for 24 h. The frozen enzyme preparation was dissolved in 6 volumes of 0.05 *M* potassium phosphate buffer, pH 7.5, containing $5 \cdot 10^{-5}$ *M* EDTA (ethylenediaminetetraacetic acid), and then dialyzed for 12 h against the same buffer. The small amount of protein precipitate that formed was removed by centrifugation.

The clear, yellowish solution (4 ml containing about 40 mg of protein) was slowly pipetted into the column. It was further allowed to travel, as observed by the movement of the yellow color, to a point about 17 cm from the top of the column by the gradual addition of the phosphate buffer. After setting the column into the electrophoresis apparatus, the two limbs of the assembly were dipped into the electrode vessels containing the same buffer. The power supply was adjusted to 750 V, allowing approximately 50 mA of current to flow for 16 h. At the end of the run, the column was removed, a stopcock was attached and it was placed over a fraction collector and the contents were eluted with the same phosphate buffer at a flow rate of approx. 18 to 20 ml/h. 25 fractions were collected of about 3 ml each. The whole operation was carried out at about 6°.

The protein content and serine deaminase activity of the fractions were then determined, and the results plotted (Fig. 1). It was observed that the highest enzymic activity was always found in the fractions which were the deepest yellow in color, which aided in their location. This color was found to travel together with the enzyme protein. The L-serine deaminase purified in this manner attained a specific activity of 346 units/mg of protein.

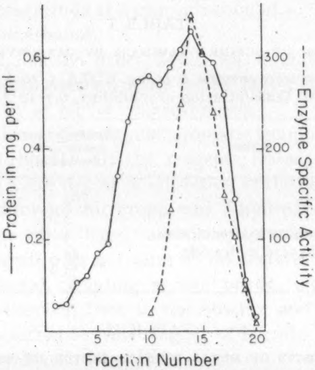

Fig. 1. Partition of protein and enzyme activity of cystathionine synthetase-serine deaminase on column electrophoresis.

In a second run performed in exactly the same way, a specific activity of 334 serine deaminase units/mg of protein was obtained. The specific activity of cystathionine synthetase, determined on the fraction with the highest serine deaminase activity, was 121. This gives a ratio for serine deaminase to cystathionine synthetase activities of 2.76, which is in good agreement with the previous findings[1].

For the additional studies reported here, batches of 100 to 120 mg of enzyme protein were fractionated by electrophoresis and those of highest enzyme activity collected. The combined fractions were brought to 50 % saturation with ammonium sulfate by the slow addition of solid salt while stirring the mixture mechanically and periodically adjusting the pH to 7.2 with ammonium hydroxide. After standing for 6 h at 0°, the mixture was centrifuged at 8,000 × *g* and the resulting precipitate stored at —20°.

Effect of various inhibitors on the serine deaminase activity of the enzyme

Effect of pyridoxine derivatives: Addition of pyridoxal phosphate to the incubation media appreciably increased the enzyme activity[1]. It was of interest therefore to determine the effect of pyridoxine derivatives on the enzyme activity. Tests were made with 4-deoxypyridoxine-5-phosphate, 4-deoxypyridoxine hydrochloride, and pyridoxine hydrochloride at a concentration of $4 \cdot 10^{-3}\,M$ on serine deaminase activity in the presence of $5 \cdot 10^{-5}\,M$ pyridoxal phosphate. The inhibitory effect found was small, amounting to only 10 to 15 %.

Inhibition by carbonyl reagents: The effect of a number of carbonyl reagents on serine deaminase activity are summarized in Table I. In this experiment, a second control was also run in the same manner as for the enzyme assay, except that the carbonyl reagent was added after the incubation, followed by the addition of trichloroacetic acid. A correction for any action of the carbonyl reagent on the development of the color of the pyruvyl-2,4-dinitrophenylhydrazone was thus determined and applied to the enzyme results. All carbonyl reagents inhibited the enzyme activity, decreasing in effectiveness in the order; hydroxylamine > cyanide > isonicotinic acid hydrazide > sodium bisulfite.

TABLE I

INHIBITION OF SERINE DEAMINASE BY CARBONYL REAGENTS

Components not listed in text were enzyme, 0.45 mg, EDTA, $5 \cdot 10^{-4}$ *M*; mercaptoethanol, $1.3 \cdot 10^{-4}$ *M*. Concentration of inhibitors, $6.7 \cdot 10^{-3}$ *M*.

Inhibitor	*Enzyme activity µmoles serine deaminated*	*Inhibition per cent*
None	65	
NaCN	5.6	91
Hydroxylamine hydrochloride	4	94
Isonicotinic acid hydrazide	44	32
$NaHSO_3$	52.2	20

TABLE II

EFFECTS OF METAL IONS ON SERINE DEAMINASE

Enzyme protein 0.36 mg. Concentration of each metal ion, $3.3 \cdot 10^{-4}$ *M*.

Metal ions added	*Enzyme activity µmoles serine deaminated*	*Per cent change*
None	52.3	
Cu^{++}	4.2	−92
Hg^{++}	1	−98
Cd^{++}	1.6	−97
Fe^{++}	47.7	−13
Co^{++}	51	−7
Ni^{++}	48.8	−12
Mn^{++}	54.6	+4
Zn^{++}	53	+1
Mg^{++}	57	+9

Action of sulfhydryl reagents: Determination of the effect of *p*-chloromercuribenzoate, N-ethylmaleimide, *o*-iodosobenzoate and iodoacetate on serine deaminase activity at approximately 10^{-3} *M*, yielded the following result. The first three strongly inhibited the enzymic activity (80–100%); iodoacetate inhibited only moderately (37%).

Inhibition by metal ions: Table II summarizes the effect of various metal ions on serine deaminase activity. Controls which differed from the enzyme assay only in that the enzyme preparation was added after incubation were run with each assay. With the exception of Mg^{++}, Mn^{++} and Zn^{++}, all of the metal ions tested inhibited the enzymic activity. The most effective inhibitors were Hg^{++}, Cd^{++} and Cu^{++} which produced almost complete inhibition at the concentration employed. Mg^{++} and Mn^{++} caused a small but significant activation of the serine deamination reaction.

Effect of metal chelating and related compounds: It was of interest to determine the effect of varying concentrations of EDTA, mercaptoethanol and dimercaptopropanol on serine deaminase activity in the presence of pyridoxal phosphate because of their use as components of the incubation media. Both EDTA and mercaptoethanol stimulated the enzymic activity slightly at the molar concentrations of $5 \cdot 10^{-4}$ and $1.3 \cdot 10^{-4}$ respectively. At higher concentrations both substances become inhibitory. The inhibition increases gradually with increasing concentration of the reagent, and becomes of considerable magnitude at a concentration of 10^{-2} *M* (—18.5 % for EDTA and —11.5 % for mercaptoethanol).

Dimercaptopropanol, on the other hand, was found to inhibit the enzymic activity even at the lowest concentration tested ($1.3 \cdot 10^{-4}$ *M*), and the degree of inhibition increased greatly at higher concentrations.

The effects of a number of heterocyclic aromatic compounds on serine deaminase activity are shown in Table III. Of the reagents tested, 8-hydroxyquinoline and *o*-phenanthroline showed a low degree of inhibition at the concentration of $4 \cdot 10^{-3}$ *M*. The most effective inhibitor in this group was 8-hydroxyquinoline-5-sulfonic acid, (30 % inhibition). On the other hand, 2-hydroxyquinoline produced no inhibition possibly because this reagent does not form metal chelates[4].

Unlike the other metal chelating agents tested, 2,2′-bipyridine appreciably stimulated the enzymic reaction both in the absence and presence of EDTA. This may be attributed to chelation by this reagent of harmful metal ions such as those of iron or copper, thereby inactivating them.

TABLE III

EFFECT OF CHELATING AGENTS ON SERINE DEAMINASE ACTIVITY

Enzyme protein 0.37 mg; EDTA, $5 \cdot 10^{-4}$. Concentration of chelating agents, $4 \cdot 10^{-3}$ *M*.

Compound added	*Enzyme activity µmoles serine deaminated*	*Per cent change*
None	53	
8-hydroxyquinoline	46.2	— 13
o-phenanthroline	50	— 6
2-hydroxyquinoline	53	0
8-hydroxyquinoline-5-sulfonic acid	37.2	— 30
2,2′-bipyridine	62.2	+ 17

Substrate specificity of serine deaminase-cystathionine synthetase

The relative activities of the enzyme on a number of hydroxy and S-containing amino acids and alanine are summarized in Table IV. The enzyme action on the amino acids was determined by the formation of α-keto acid. Lithium pyruvate was used as the color standard, regardless of the nature of the α-keto acid expected to be produced from a given amino acid.

Of the hydroxyamino acids tested, L-threonine was deaminated at about 20 % of the rate of L-serine. Possibly this activity may reflect contamination of the enzyme with L-threonine deaminase, also present in rat liver, but this cannot be accepted

TABLE IV

ACTIVITY OF SERINE DEAMINASE ON HYDROXY AND SULFUR-CONTAINING AMINO ACIDS

Enzyme protein, 0.36 mg; EDTA, $5 \cdot 10^{-4}$ *M*; mercaptoethanol, $1.3 \cdot 10^{-4}$ *M*.

Amino acid	*Amount added μmoles*	*Enzyme activity μmoles serine deaminated*
L-serine	200	57
L-threonine	200	11.4
L-homoserine	200	0.6
β-hydroxy-DL-aspartic acid*	100	0
D-glucosamine hydrochloride	200	1.1
DL-homocysteine	250	0.5
L-cysteine	200	0
DL (+ allo)-cystathionine	50	0
Lanthionine (L and meso)	50	0.6
S-isopropyl-DL-homocysteine	50	0
S-methyl-L-cysteine	100	0.2
S-carboxylmethyl-L-cysteine	50	0.6
DL-ethionine	100	0.4
L-methionine	100	1.0

* A synthetic preparation kindly furnished by Dr. E. KUN. Probably composed of mixture of the several possible configurations.

with certainty. The other hydroxyamino acids, and also the sulfur-containing amino acids tested were found to be essentially inert as substrates for the enzyme.

Activity of enzyme on L-serine in presence of certain sulfur-containing amino acids.

The effect of certain sulfur-containing amino acids on the serine deaminase and apparent cystathionine synthetase activity of the enzyme is recorded in Table V. The synthetase activity of the enzyme was evaluated by determining the amount of L-serine which reacted but was not converted to pyruvic acid, as described in the previous communication[1].

The results obtained with homocysteine and cysteine parallel those already published. Both of these amino acids virtually eliminate serine deaminase activity, but only homocysteine induces the synthetase reaction.

Substitution of the sulfhydryl group of these amino acids greatly reduced the

TABLE V

ACTIVITY OF SERINE DEAMINASE AND CYSTATHIONINE SYNTHETASE ON SULFUR CONTAINING AMINO ACIDS IN PRESENCE OF L-SERINE

Enzyme protein, 0.36 mg: mercaptoethanol, $1.3 \cdot 10^{-4}$; EDTA, $5 \cdot 10^{-4}$ *M*; 200 μmoles of L-serine and the sulfur amino acid in the amounts shown below.

Sulfur amino acid	*Amount added μmoles*	*Serine deaminase activity μmoles serine utilized*	*Cystathionine synthetase activity μmoles serine utilized*
None		58	
DL-homocysteine	250	1.3	21.5
L-cysteine	200	1.1	0
S-isopropyl-DL-homocysteine	100	45·7	3·3
S-methyl-L-cysteine	100	35	2.4

degree of inhibition of serine deaminase activity. Both *S*-isopropyl-DL-homocysteine and *S*-methyl-L-cysteine caused some apparent synthetase activity. For the former, this might be explained by some contamination or decomposition resulting in the occurrence of homocysteine. The explanation of the result indicating a synthetic effect of *S*-methyl-L-cysteine is not readily apparent.

Activity of the enzyme on hydroxyamino acids in the presence of homocysteine

The relative synthetase activity of the enzyme was tested on a number of hydroxyamino acids, in the presence of DL-homocysteine (Table VI). Synthetic activity was estimated by the disappearance of homocysteine from the reaction mixtures by means of the nitroprusside method of GRUNERT AND PHILIP[5]. Two controls were run simultaneously with the enzyme assay; in one, the enzyme preparation was added at the end of the incubation; in the second the hydroxyamino acid was added after incubation.

TABLE VI

ACTIVITY OF CYSTATHIONINE SYNTHETASE ON HYDROXYAMINO ACIDS IN PRESENCE OF HOMOCYSTEINE

Enzyme protein, 0.36 mg; EDTA, $5 \cdot 10^{-4}$ *M*; mercaptoethanol, $1.3 \cdot 10^{-4}$ *M*; 250 µmoles DL-homocysteine.

Hydroxyamino acid added	*Amount added µmoles*	*Cystathionine synthetase activity µmoles homocysteine utilized*
L-serine	200	21
L-threonine	200	0.45
Hydroxy-DL-aspartic acid	100	0.3
L-homoserine	200	0.7

The results indicate that, with the exception of L-serine which is a highly potent substrate for synthetic activity, all of the other amino acids were not utilized by the enzyme.

The results reported in this communication support the previous conclusion[1] that dehydrative deamination of serine and synthesis of cystathionine are functions of a single enzyme protein. Additional evidence has been obtained that pyridoxal phosphate is the coenzyme for the enzyme activities and that the enzyme requires free sulfhydryl groups for activity.

ACKNOWLEDGEMENT

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REFERENCES

[1] A. S. M. SELIM AND D. M. GREENBERG, *J. Biol. Chem.*, 234 (1959) 1474.
[2] J. PORATH, *Acta Chem. Scand.*, 8 (1954) 1813.
[3] L. C. MOKRASCH AND R. W. MCGILVERY, *J. Biol. Chem.*, 221 (1956) 909.
[4] A. ALBERT AND D. MAGRATH, *J. Biochem.*, 41 (1947) 534.
[5] R. R. GRUNERT AND P. H. PHILILPS, *Arch. Biochem.*, 30 (1951) 217.

THE *IN VIVO* INCORPORATION OF [^{32}P] INORGANIC PHOSPHATE INTO LOMBRICINE AND SERINE ETHANOLAMINE PHOSPHODIESTER OF THE EARTHWORM

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SUMMARY

1. At time intervals varying from one to 16 days after the administration of [^{32}P]inorganic phosphate to earthworms the specific radioactivity of serine ethanolamine phosphodiester (SEP) was greater than that of lombricine in both muscle and gut tissue.

2. In gut the specific radioactivity of both SEP and lombricine was greater than that of the corresponding compounds in muscle at all time intervals after the administration of ^{32}P.

3. In muscle the concentration of lombricine was much greater than that of SEP. In gut the concentrations of these compounds were of the same order and similar to that of SEP in muscle.

4. The incorporation of [^{32}P]inorganic phosphate into the labile P of both ATP and N-phosphoryllombricine was considerably more rapid than the incorporation into the diester P of SEP and lombricine.

5. The rate of incorporation of [^{32}P]inorganic phosphate into SEP was similar to that found for phospholipid and phosphoprotein.

6. It is concluded that in the earthworm SEP is formed from smaller molecules and is the precursor of lombricine.

INTRODUCTION

Serine ethanolamine phosphodiester (SEP, I) has been recently isolated from and identified in the earthworm[1, 2], a finding which is consistent with the previous suggestion[2, 3] that this compound is the biological precursor of lombricine (II), the N-phosphoryl derivative of which was claimed to be the phosphagen of this species[4, 5].

The suggestion that SEP gives rise to lombricine was further strengthened by the finding that earthworm SEP contains the unusual D-isomer of serine[2], which is also present in lombricine[6]. The present communication offers additional support of this hypothesis from evidence from experiments on the *in vivo* incorporation of [^{32}P]-orthophosphate into SEP and lombricine in the earthworm. In addition, experiments are reported in which the relatively rapid incorporation of [^{32}P]inorganic phosphate into the labile P of both ATP and N-phosphoryllombricine is contrasted with the very slow incorporation into the diester P of SEP and lombricine.

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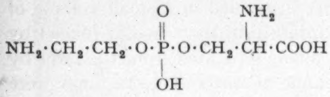
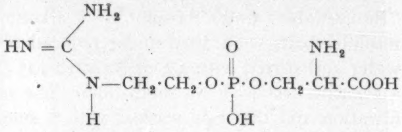


$$NH_2{\cdot}CH_2{\cdot}CH_2{\cdot}O{\cdot}\underset{OH}{\overset{\overset{O}{\|}}{P}}{\cdot}O{\cdot}CH_2{\cdot}\overset{NH_2}{CH}{\cdot}COOH$$

(I)



(II)

EXPERIMENTAL

Materials

All reagents were analytical grade and solvents used for chromatography were redistilled before use. Carrier free [^{32}P]inorganic phosphate was obtained from the Atomic Energy Research Establishment, Harwell, England.

Treatment of worms

Earthworms (*Megascolides cameroni*) of average weight 8 g were collected in Australian Capital Territory and were kept at 10° in moist earth taken from their natural habitat. [^{32}P]Inorganic phosphate (10 μC/g) was administered from a microsyringe fitted with fine polythene tubing[7], which was inserted into the gastrointestinal tract for a distance of at least 3 cm. During the incubation procedure the worms were lightly anaesthetised with CO_2. After intubation the worms were returned to the cold room (10°) for periods varying from 24 h to 16 days.

Preparation of tissue extracts

The worms were killed and the gut, together with other viscera, dissected free from the muscular body wall. These two tissues were then homogenized at 0° with two volumes of 1.5 *N* perchloric acid, and the protein precipitate removed by centrifugation. The supernatant was neutralised with 2.5 *N* KOH at 0° and the precipitated $KClO_4$ centrifuged off. Glycogen was removed from the supernatant by treatment with an equal volume of ethanol at 0° and subsequent centrifugation. A portion of the supernatant was taken for the estimation of the specific radioactivity of inorganic phosphate. The remainder was applied to a column (6×1 cm) of Zeokarb 225 ion exchange resin (H^+ form) at a rate of 1 ml/min. Phosphoric acid (0.01 *N*) was then passed through the column to remove traces of [^{32}P]inorganic phosphate remaining in the column. This was followed by 0.01 *N* HCl until the effluent was free from inorganic phosphate. The material retained on the resin was eluted with 2.5 *N* ammonium hydroxide (rate 3 ml/min) and the eluate (approx. 50 ml) evaporated to dryness under reduced pressure at 40°. The residue was dissolved in a small volume of distilled water (100 μl and 300 μl in the case of gut and muscle, respectively).

In some experiments the entire worm was frozen in liquid air, ground to a fine powder in a cooled mortar and extracted with cold perchloric acid as before. The extract was clarified by centrifugation at 0° and immediately adjusted to pH 7.2 by the addition of 2.5 *N* KOH. The precipitated $KClO_4$ was removed by centrifugation at 0° and samples taken from the supernatant for the isolation of SEP and lombricine for the determination of specific radioactivities as described below.

Another sample of the neutralised extract was taken for separation into the "Ba-insoluble" and "Ba-soluble" P fractions as described by Le Page[8]. The Ba-insoluble salts were dried under reduced pressure, suspended in a small volume of water and stirred with 2 g of Zeokarb-225 (Na^+ form) until Ba^{++} free as judged by a negative test with Na rhodizonate. The solution was separated from the resin by filtration and the resin washed with a small volume of water. The washings were combined with the filtrate and samples from the resultant solution taken for determination of the specific radioactivities of inorganic phosphate, and the P released as a result of hydrolysis in *N* HCl for 7 min at 100° (P_7)[9]. This latter P fraction has been taken to represent primarily the labile P of ATP.

The "Ba-soluble" fraction was isolated from the supernatant after removal of the Ba-insoluble salts by the addition of four volumes of ethanol. It was dried under reduced pressure, redissolved in water and reprecipitated by the addition of ethanol. The reprecipitated material was dissolved in water and converted to the Na form by passage through a column (1 × 5 cm) of Zeokarb-225 (Na^+ form) ion exchange resin. Samples were removed from the resultant solution for determination of the specific radioactivities of residual inorganic phosphate, and P released (P_9) by hydrolysis for 9 min at 65° in 0.1 *N* HCl (*cf.* Ennor and Rosenberg[9]).

In addition to determination of the amount of P released by such treatment, estimations were made of the amount of lombricine released. The molar ratio P: lombricine varied from 0.97 to 1.01 and thus the P_9 was assumed to have its origin in the phosphoryl group of N-phosphoryllombricine.

The specific radioactivities of the phospholipids and phosphoprotein P were determined after fractionation of the acid-insoluble material by a procedure similar to that of Schmidt and Thannhauser[10]. The acid-extracted material was washed repeatedly with *N* $HClO_4$ at 0° until the wash no longer showed radioactivity[11]. The pellet was then extracted with lipid solvents as described by Schmidt and Thannhauser[10], and dried under reduced pressure. The fat-free material was hydrolysed in *N* KOH at 37° for 16 h and the inorganic phosphate precipitated according to Delory[12].

The lipid extracts were combined and dried under reduced pressure at 40°. The lipid was re-extracted with petroleum ether (boiling range: 40°–60°) and the volume of the extract was reduced to 1–2 ml. Acetone (20 ml) was then added, followed by 2 drops of a saturated solution of $MgCl_2$ in ethanol. The precipitated phospholipids were collected by centrifugation, dried and wet ashed.

Chromatography

The entire sample of the concentrated, de-salted gut extract, and 100 μl of the corresponding muscle extract, were used for two-dimensional chromatography (descending) on Whatman No. 3 paper (56 × 56 cm). The solvent pair employed for the separation of lombricine, SEP and the free amino acids was ethanol–formic acid–water (70:10:20, v/v) followed by phenol–water (4:1, w/v). In addition to these, other standard solvent systems were used for the identification of the amino acids referred to in Figs. 1 and 2.

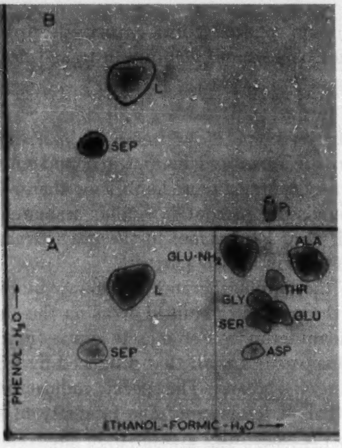


Fig. 1. A. Chromatogram of extract of earthworm muscle prepared 8 days after the administration of [^{32}P]inorganic phosphate ($1.1 \cdot 10^6$ counts/min/g body weight). SEP, serine ethanol amine phosphodiester; L, lombricine; Glu-NH$_2$, glutamine; Ala, alanine; Thr, threonine; Gly, glycine; Glu, glutamic acid; Ser, serine; Asp, aspartic acid. B. Radioautograph of chromatogram shown in A. P$_I$, inorganic P. The lines encircling the ninhydrin-stained areas of SEP and lombricine (L) on the chromatogram were superimposed on the radioautograph.

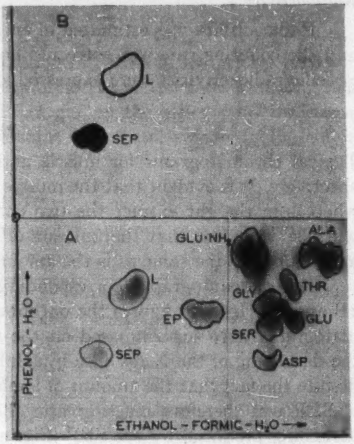


Fig. 2. A. Chromatogram of extract of earthworm gut prepared 8 days after the administration of [^{32}P]inorganic phosphate ($1.1 \cdot 10^6$ counts/min/g body weight). Abbreviations as in Fig. 1. B. Radioautograph of chromatogram shown in A. Abbreviations as in Fig. 1. The lines encircling the ninhydrin-stained areas of SEP and lombricine (L) on the chromatogram were superimposed on the radioautograph.

Chromatograms were developed by spraying with a 0.2 % (w/v) solution of ninhydrin in acetone and heating at 70° for 10 min.

Radioautography

Radioautographs were obtained by exposing Ilford Industrial G film to the chromatograms for 14 days.

Analytical methods

Lombricine was estimated before and after hydrolysis by the procedure described by ROSENBERG, ENNOR AND MORRISON[13] and the specific radioactivity of inorganic phosphate (P$_I$) according to ENNOR AND ROSENBERG[9].

In order to estimate the amounts and specific radioactivity of P in SEP and lombricine the areas containing these compounds were cut from the developed chromatograms and the compounds eluted with water. The eluates were taken to dryness and the residue ashed with 0.5 ml of a mixture of 2 volumes of 70 % perchloric acid and 3 volumes of concentrated H_2SO_4. The ashed residue was diluted with 2 ml of water, heated at 100° for 1 h to convert any pyrophosphate into orthophosphate and the specific radioactivity determined as described above.

Radioactivity

Radioactivity was estimated in an M6 liquid counter (20th Century Electronics) and the counting rate was corrected both for background counting rate and decay. Specific radioactivities were expressed as counts/min/μmole.

RESULTS

Typical chromatograms for muscle and gut are presented in Figs. 1A and 2A respectively. It is evident that the muscle extract contains more lombricine than SEP, whereas in the gut extract the two compounds are present in similar amounts. It will also be noted that the amount of SEP (and lombricine) in the gut extract is approximately the same as is the amount of SEP in muscle extract.

The radioautographs corresponding to the chromatograms are shown in Figs. 1B and 2B. In both figures the outlines of the ninhydrin-stained areas on the chromatograms were superimposed on the radioautographs. For muscle tissue (Fig. 1) the darkening of the X-ray film by lombricine was less than that produced by SEP, despite the fact that the amount of lombricine was greater. The specific radioactivity of SEP was therefore much greater than that of lombricine. The intensity of the darkening produced by gut SEP (Fig. 2) was considerably greater than that due to muscle SEP (Fig. 1). Since these two compounds were present in similar amounts, it was concluded that the specific radioactivity of gut SEP was greater than that of muscle SEP.

The labelling of gut lombricine is not shown in the reproduction of the radioautograph, but in the original X-ray film a definite darkening could be seen. A faint spot due to [^{32}P]inorganic phosphate not completely removed in the preparative procedure is visible in the muscle radioautograph. No other radioactive areas were detected.

The above findings are substantiated by the quantitative data (Table I). Whereas the specific radioactivity of the gut inorganic P fell progressively, there was no significant change in the specific radioactivity of the muscle inorganic phosphate after the second day. The specific radioactivities of both SEP and lombricine in muscle

TABLE I

INCORPORATION OF [^{32}P]INORGANIC PHOSPHATE INTO SEP AND LOMBRICINE OF MUSCLE AND GUT OF THE EARTHWORM

Radioactivity administered, $1.1 \cdot 10^6$ counts/min/g body weight.

Time after ^{32}P (days)	*Specific radioactivity (counts/min/μmole)*					
	Muscle			*Gut*		
	P_i	*SEP*	*Lombricine*	*P_i*	*SEP*	*Lombricine*
1	17,000	270	11	56,700	2,190	40
2	11,700	560	20	29,500	4,310	160
4		840	97		4,990	100
9	12,900	2,140	140	13,400	10,500	470
16	13,000	2,640	250	10,600	7,160	310

rose progressively with time, but the specific radioactivity of SEP was at all times much greater than that of lombricine. The specific radioactivities of both SEP and lombricine in gut were at all times greater than those of the corresponding compounds in muscle, and again the specific radioactivity of SEP was greater than that of lombricine.

Quantitative estimation of the concentration of SEP and lombricine present in muscle and gut indicates (Table II) that in muscle the concentration of lombricine was considerably greater than that of SEP. In gut on the other hand the concentration of SEP and lombricine were of the same order, and similar to the concentration of SEP present in muscle.

TABLE II

CONCENTRATION OF SEP AND LOMBRICINE IN MUSCLE AND GUT OF THE EARTHWORM

μmoles/100 g tissue.

Muscle		*Gut*	
SEP	*Lombricine*	*SEP*	*Lombricine*
15.3	176	10.6	23.8

Some experiments have been carried out in which the relative rates of ^{32}P incorporation into inorganic phosphate, the labile P of ATP (P_7), the labile P of N-phosphoryllombricine (P_9), phosphoprotein and phospholipid P also were determined (Table III).

TABLE III

INCORPORATION OF [^{32}P]INORGANIC PHOSPHATE INTO CERTAIN PHOSPHORUS-CONTAINING COMPOUNDS OF THE EARTHWORM

Radioactivity administered, $1.1 \cdot 10^6$ counts/min/g body weight.

Time after ^{32}P (days)	*Relative specific activity (based on P_i = 100)*					
	P_7 (labile P of ATP)	*P_9 (labile P of N-phosphoryl-lombricine)*	*SEP (diester P)*	*Lombricine (diester P)*	*Phosphoprotein (DELORY[12])*	*Phospholipid P (lipid sol.)*
2	23	—	0.44	0.012	4.5	0.8
2	78	53	0.86	0.073	—	—
2	44.5	36.2	3.98	0.19	—	—
4	173	175	—	0.26	—	—

After two days the relative specific radioactivities of P_7 and P_9 were similar, but less than that of the inorganic P. After four days, although these specific radioactivities were still similar to each other, they were greater than that of inorganic phosphate.

The specific radioactivities of SEP, lombricine, phosphoprotein and phospholipid P were all considerably less than that of the labile P. Whereas the specific radioactivity of SEP was of the same order as that of phosphoprotein and phospholipid P, that of lombricine was much less, in confirmation of the results reported in Table I.

DISCUSSION

The results of the present experiments show that [^{32}P]inorganic phosphate is incorporated into the diester P of both SEP and lombricine. The degree to which such incorporation occurred is consistent with the hypothesis that SEP is a precursor of lombricine in the earthworm and not a degradation product of that substance. This conclusion stems from the fact that the amount of ^{32}P incorporated into lombricine was, at each of the time intervals investigated, less than 0.1 of that which was found in the SEP.

In similar experiments with reptiles AYENGAR AND ROBERTS[14] were unable to detect any incorporation of [^{32}P]inorganic phosphate into muscle SEP, but low levels of radioactivity were reported to be present in kidney SEP 48–72 h after injection. On the basis of these results these workers have concluded that ^{32}P was incorporated very slowly into SEP and that this compound was not synthesised from low molecular weight precursors. Such a finding is in contrast with the present results, where incorporation of [^{32}P]inorganic phosphate occurred in both the muscle and gut of the earthworm. The present findings are also supported by other experiments from this laboratory[15] with [^{14}C]serine, [^{14}C]ethanolamine and [^{3}H]serine, which afford further evidence that in the earthworm SEP is synthesised from low molecular weight precursors.

Some comparison of the metabolic activities of reptilian and earthworm SEP may be obtained from the relative rates of ^{32}P incorporation into SEP and phospholipid P in each species. According to AYENGAR AND ROBERTS[14], reptilian SEP contained no detectable label at the time when phospholipid P attained considerable radioactivity. On the other hand, in the earthworm the labelling of SEP was similar to that of the phospholipid (*cf.* Table III).

There is thus a difference in the relative rates of formation of SEP and phospholipid in the two species. It should, however, be pointed out that SEP in the reptilian muscle contains the L-isomer of serine[16], while in the earthworm the D-enantiomorph[2] is present. It is probable, therefore, that SEP has a different function in each of these species—in fact, one function of earthworm SEP is to give rise to lombricine. This is not shared by reptilian SEP, for lombricine was not detected in any of the reptiles studied (unpublished observations).

The size of the constituent organs of the earthworm has precluded more than a rough breakdown into muscle and gut, which latter comprises gastro-intestinal tract, gonads, pseudo-hearts, etc. Nevertheless large differences in the specific radioactivities of the diester P of SEP and lombricine have been found as between these two tissues and in all cases the higher values were found in the compounds isolated from the gut. It may be concluded therefore that SEP is formed in the gut.

The rate of incorporation of ^{32}P into SEP (and lombricine) is extremely slow compared to the rate of incorporation of this isotope into the labile P of ATP and is indicative of a very slow turnover rate of SEP in the earthworm. The present experiments do not give any precise indication of the speed with which the labile P of ATP or the phosphoryl group of N-phosphoryl lombricine is replaced. It is however of some interest to note that the relative specific radioactivities of the labile P of ATP and N-phosphoryl lombricine are approximately equal—such a result would be expected, for the relationship between these groups is determined by a phosphoryltransferase, some properties of which have recently been described[17,18].

REFERENCES

[1] H. ROSENBERG AND A. H. ENNOR, *Biochem. J.*, 73 (1959) 521.
[2] A. H. ENNOR, H. ROSENBERG, R. J. ROSSITER, I. M. BEATTY AND T. J. GAFFNEY, *Biochem. J.*, 75 (1960(179.
[3] A. H. ENNOR AND J. F. MORRISON, *Physiol. Rev.*, 38 (1958) 631.
[4] N. v. THOAI, J. ROCHE, Y. ROBIN AND N. V. THIEM, *Compt. rend. soc. biol.*, 147 (1953) 1670.
[5] N. v. THOAI AND Y. ROBIN, *Biochim. Biophys. Acta*, 14 (1954) 76.
[6] I. M. BEATTY, D. I. MAGRATH AND A. H. ENNOR, *Nature*, 183 (1959) 591.
[7] S. COHEN AND H. B. LEWIS, *J. Biol. Chem.*, 180 (1949) 79.
[8] G. A. LE PAGE, in W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques and Tissue Metabolism*, 1951, p. 185.
[9] A. H. ENNOR AND H. ROSENBERG, *Biochem. J.*, 51 (1952) 606.
[10] G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
[11] A. H. ENNOR AND H. ROSENBERG, *Australian J. Exptl. Biol. Med. Sci.*, 32 (1954) 701.
[12] G. E. DELORY, *Biochem. J.*, 32 (1938) 1161.
[13] H. ROSENBERG, A. H. ENNOR AND J. F. MORRISON, *Biochem. J.*, 63 (1956) 153.
[14] P. AYENGAR AND E. ROBERTS, *Federation Proc.*, 16 (1957) 147.
[15] R. J. ROSSITER, T. J. GAFFNEY, H. ROSENBERG AND A. H. ENNOR, *Nature*, 185 (1960) 383.
[16] E. ROBERTS AND I. P. LOWE, *J. Biol. Chem.*, 211 (1954) 1.
[17] H. ROSENBERG, R. J. ROSSITER, T. GAFFNEY AND A. H. ENNOR, *Biochim. Biophys. Acta*, 37 (1960) 385.
[18] R. PANT, *Biochem. J.*, 73 (1959) 30.

STUDIES ON THE INCORPORATION OF THYMIDINE INTO DNA BY RAT-LIVER HOMOGENATES *IN VITRO*

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SUMMARY

$[^3H]$thymidine is incorporated into DNA by homogenates of normal and 24 h regenerating liver. Regenerating liver homogenates are somewhat more active than normal liver homogenates. The incorporation of thymidine is not inhibited by ethionine, *p*-fluorophenylalanine, or β-2-thienylalanine when these compounds are added to incubation flasks in solid form. However, chloramphenicol inhibits incorporation by 19 to 91 % at concentrations ranging from 0.38 mM to 6.21 mM.

The ability of both normal and regenerating liver homogenates to incorporate thymidine into DNA is markedly inhibited by the injection of ethionine 8–14 h before the liver is excised. This suggests that the injected ethionine prevents the synthesis of an enzyme necessary for thymidine incorporation, or inhibits its activity.

INTRODUCTION

It has been demonstrated that the incorporation of $[^3H]$thymidine into regenerating rat liver DNA *in vivo* can be strongly inhibited by the intraperitoneal injection of

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Abbreviation: DNA, deoxyribonucleic acid.

ethionine, ethionine hydrochloride, *p*-fluorophenylalanine, and β-2-thienylalanine hydrochloride[1]. Chloramphenicol also inhibits incorporation under certain conditions. It was therefore desirable to examine the action of these compounds in more detail by using an *in vitro* system.

METHODS

The conditions used for incubation are given in the legend of Table I. Methods used for isolation of the DNA and the sources of the inhibitors tested have been given previously[1]. Rats used in these experiments were either obtained from Germany (Tierzuchterei Brunger, Halle, Westfalen, Germany) or from a local rat colony originating from the German source. All regenerating livers in these experiments were excised 24 h after partial hepatectomy.

TABLE I

THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF THYMIDINE INTO DNA

The incubation medium contained the following components at the final concentrations indicated: Glutamate, 10 m*M*; succinate, 3 m*M*; pyruvate, 10 m*M*; magnesium chloride, 3 m*M*; ATP, 0.4 m*M*; fructose, 6 m*M*; phosphate buffer, pH 7.4, 10 m*M* and sufficient sucrose to make the medium 0.25 *M* sucrose before the addition of the homogenate. The final concentration of the pooled regenerating liver homogenate (24 h after partial hepatectomy) from two male rats (Beirut) in 0.25 *M* sucrose was 10%. Each 25-ml Erlenmeyer incubation flask also contained 4 μC of [^{3}H]thymidine (Schwartz Laboratories; specific activity 360 mC/mmole) in a final volume of 4 ml. Chloramphenicol was weighed out into dry flasks before other additions, and concentrations given are those which would be present if all the chloramphenicol had dissolved. Incubation was for 45 min at 37°. The specific activities of DNA from three flasks incubated without chloramphenicol were 3010, 2830, and 2790, or an average of 2880.

Chloramphenicol ($M \times 10^{-3}$)	*Counts/min/mg DNA*	*% Inhibition*
0.00	2880	0
0.38	2340	19
0.76	1820	37
1.25	1900	34
1.63	1620	44
1.97	1560	46
2.27	1380	52
2.73	621	78
3.17	490	83
3.86	397	86
4.71	319	89
6.21	247	91

RESULTS AND DISCUSSION

When rat-liver homogenates were incubated with a mixture of cofactors, substrates, and [^{3}H]thymidine, the thymidine was incorporated into DNA. Incorporation followed a definite time course. In one experiment, for example, the specific activity of the DNA after 0, 15, 30, and 60 min of incubation was 5, 340, 950, and 1235 counts/min/mg DNA respectively. No significant radioactivity was observed in DNA isolated from flasks that were identical to the others except that they were not incubated. This result indicates that the isolation method completely removes all trace of acid-soluble compounds such as the radioactive thymidine and its phosphorylated derivatives.

When analogs of amino acids were added directly to flasks containing fortified homogenates of 24 h regenerating rat liver and incubated for 45 min, it was shown that chloramphenicol produced an inhibition of approximately 75 %. Ethionine, *p*-fluoro-phenylalanine and β-2-thienylalanine did not show any significant inhibition. In each case, 4 mg of the analog was added in solid form to flasks prepared as described in Table I. The failure to observe inhibition with analogs other than chloramphenicol may be due to the fact that these compounds are not very soluble in water. Another possible explanation is that these compounds have no direct inhibitory effect on the enzymes concerned with thymidine incorporation, but that inhibition observed *in vivo*[1] is due to some other action, such as inhibition of enzyme synthesis.

The inhibition of thymidine incorporation by chloramphenicol was examined in more detail by correlating the concentration of chloramphenicol with the per cent inhibition as shown in Table I. It may be seen that inhibition ranging from 19 to 91 % can be obtained with chloramphenicol at concentrations of 0.38 to 6.21 m*M*. The only definitely established primary effect of chloramphenicol at a metabolic level is the inhibition of protein synthesis although the effect on other reactions has been rather extensively investigated[2,3]. It is known that in some cases chloramphenicol inhibits the uptake of precursors into nucleic acids[4–8] while in other cases nucleic acid synthesis is stimulated or not affected[9–13]. It has not been established that the effect on nucleic acid synthesis (when it is observed) is due only to the inhibition of protein synthesis.

The specific activities of DNA obtained after incubation of [^{3}H]thymidine with normal and regenerating rat-liver homogenates are presented in Table II as the "control" values. It may be seen that there is a wide variation in the ability of homogenates to incorporate thymidine into DNA, but that the regenerating liver homogenates appear to be approximately twice as active as the homogenates of normal liver, if mean values are compared.

The reason for variation from rat to rat and from experiment to experiment has not yet been determined. The values presented in Table II are the average of results from duplicate or triplicate flasks containing aliquots of the same homogenate. Values for these duplicate analyses rarely varied more than ± 8 % from the average, indicating that the variation observed is not due to errors of incubation, isolation of DNA or determination of specific activity. The extent of homogenization within wide limits does not have a significant effect on the incorporation. In addition, aging the homogenate for 1 h in an ice bath produces only a very slight increase in incorporation. A more significant stimulation (60 %) is obtained by aging the homogenate for 4 h.

The incubation medium used in these experiments is one that was found to be satisfactory for the incorporation of orotic acid into RNA[14]. BOLLUM AND POTTER[15] have also published a note on the incorporation of thymidine into normal and regenerating liver homogenates using a sucrose system that differs in several respects from the one used in this work. In their experiment, BOLLUM AND POTTER observed that a regenerating rat-liver homogenate showed 44 times as much activity as a normal rat-liver homogenate. It is not yet known why slight differences in the incubation conditions produce this difference in results.

TABLE II

THE EFFECT OF ETHIONINE INJECTION ON THE INCORPORATION OF THYMIDINE INTO DNA OF NORMAL AND REGENERATING RAT-LIVER HOMOGENATES

Rats in the "control" columns received no injection. Rats in the "ethionine" columns received one intraperitoneal injection of ethionine (0.5 mg/g body weight) at times ranging from 8–14 h before the time of sacrifice. One rat with a regenerating liver (specific activity of 155) received an injection of 1 mg of ethionine hydrochloride/g body weight. The conditions of incubation are given in Table I. Each value represents the results obtained with a homogenate from one rat, except for one value (specific activity of 258) where a pooled homogenate was used. In most cases, the value is the average specific activity of DNA obtained from duplicate or triplicate flasks containing the same homogenate. These data represent the results of 9 experiments in which both males and female rats over a wide weight were used. Although the females tended to give DNA with a lower specific activity, this trend was not clear cut, and the data were not strictly comparable since male and female rats were never tested in the same experiment under exactly identical conditions. Incubation was for 45 min at 37° for most experiments, but in several cases, 60 min incubation times (indicated by parantheses in the Table) were used.

Counts/min/mg DNA			
Normal liver		*Regenerating liver*	
Control	*+ Ethionine*	*Control*	*+ Ethionine*
296	33	(295)	75
345	41	(470)	83
378	48	673	131
392	55	695	155
425	73	895	212
(480)		1010	258
(548)		1020	286
635		(1520)	*
(785)		1580	
911		1660	
(1060)		(2530)	
(1240)		2880	
(1790)		3870	
548**	48**	1020**	155**

* One value of 408 has been omitted since the colorimetric determination of DNA in duplicate samples gave values which were unaccountably low, indicating a procedural error.

** Mean values for the column.

It is known that the intraperitoneal injection of ethionine at a level of 0.5 mg/g body weight inhibits the incorporation of $[^3H]$thymidine into DNA by 97 %[1]. This inhibition was examined in more detail by incubating $[^3H]$thymidine with liver homogenates obtained from rats injected with ethionine (see Table II). If the mean values of injected and uninjected rats are compared, it may be seen that the ethionine injection has inhibited the thymidine incorporation in normal and regenerating liver homogenates by 91 and 84 %, respectively.

This marked inhibition of the ability of both normal and regenerating liver homogenates to incorporate $[^3H]$thymidine into DNA indicates that ethionine has prevented the synthesis or inhibited the activity of some enzyme required for thymidine incorporation. It should be of interest to determine whether it is the enzymes that phosphorylate thymidine, or the enzyme that polymerizes the deoxynucleotides to DNA, (or both) which are less active or lacking in homogenates as a result of the ethionine injection.

Deoxynucleotides other than thymidine are required as co-substrates for thymidine incorporation if a true DNA molecule is being synthesized. The possibility that ethionine might inhibit thymidine incorporation by lowering the level of these co-substrates was tested by adding deoxynucleotides of cytosine, adenine and guanine to flasks simultaneously at levels of 0.4 mg/flask in one set of four flasks and at levels of 4 mg/flask in a second series of four flasks. These flasks contained the usual incubation medium as well as normal liver homogenates from rats that had been injected with saline or with ethionine. In no case was significant stimulation of incorporation observed. Instead, in three out of four flasks at the 4 mg level, the specific activity of DNA was lower than control values by 52, 69, and 81 %. These results indicate that the injection of ethionine has not lowered the tissue levels of deoxynucleotides. Thus, the reason why injection of ethionine inhibits the incorporation of tritium-labeled thymidine into DNA *in vitro* still remains to be elucidated.

ACKNOWLEDGEMENT

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REFERENCES

[1] J. H. Schneider, R. Cassir and F. Chordikian, *J. Biol. Chem.*, 235 (1960) 1437.
[2] G. N. Smith, *Bacteriol. Rev.*, 17 (1953) 19.
[3] F. E. Hahn, C. L. Wisseman and H. E. Hopps, *J. Bacteriol.*, 69 (1955) 215.
[4] G. A. LePage, *Proc. Soc. Exptl. Biol. Med.*, 83 (1953) 724.
[5] F. Gros and F. Gros, *Biochim. Biophys. Acta*, 22 (1956) 200.
[6] G. C. Webster, *Arch. Biochem. Biophys.*, 68 (1957) 403.
[7] T. R. Breitman and G. C. Webster, *Biochim. Biophys. Acta*, 22 (1956) 200.
[8] M. Drakulic and M. Errera, *Biochim. Biophys. Acta*, 31 (1959) 459.
[9] E. F. Gale and J. P. Folkes, *Biochem. J.*, 53 (1953) 493.
[10] C. L. Wisseman, J. E. Smadel, F. E. Hahn and H. E. Hopps, *J. Bacteriol.*, 67 (1954) 662.
[11] A. B. Pardee and L. S. Prestidge, *J. Bacteriol.*, 71 (1956) 677.
[12] M. Ycas and G. Brawerman, *Arch. Biochem. Biophys.*, 68 (1957) 118.
[13] F. E. Hahn, M. Schaecter, W. S. Ceglowski, H. E. Hopps and J. Ciak, *Biochim. Biophys. Acta*, 26 (1957) 469.
[14] J. H. Schneider and V. R. Potter, *J. Biol. Chem.*, 233 (1958) 154.
[15] F. J. Bollum and V. R. Potter, *J. Am. Chem. Soc.*, 79 (1957) 3603.

Biochim. Biophys. Acta, 42 (1960) 225–229

SUR L'ACTIVATION DU TRYPSINOGÈNE DE BOEUF PAR UNE PROTÉINASE CRISTALLISÉE D'*ASPERGILLUS SAITOI*

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SUMMARY

On the activation of beef trypsinogen by a crystallized proteinase of Aspergillus saitoi

1. A proteolytic enzyme derived in crystallized form from *Aspergillus saitoi* is capable of activating trypsinogen and chymotrypsinogen-A at pH 4.5. In the case of trypsinogen, activation is rapid at 0°. Its kinetics are of the first order, and a specific activity equal to the maximum value attained by the classical method of auto-activation can be reached.

2. The mechanism of this new kind of activation appears to be the same as that of auto-activation. It comprises—like self-activation—the specific rupture of the sixth bond (Lys·Ileu) of the trypsinogen chain. Due to its higher specificity, the enzyme of *Aspergillus saitoi* seems to be capable of breaking another linkage in the trypsinogen, without, however, diminishing the final activity.

INTRODUCTION

KUNITZ a montré depuis longtemps que le trypsinogène de boeuf est activé par au moins trois enzymes différents: la trypsine (autocatalyse) à pH 7.8 (voir réf. 1, 2), l'entérokinase à pH 5.6 (voir réf. 3) et une préparation mal définie obtenue à partir d'une souche non identifiée de *Penicillium* et active à pH 3 (voir réf. 4).

Les modifications subies par la structure covalente du trypsinogène au cours de son autoactivation ont fait l'objet d'études approfondies. On sait que la trypsine coupe la 6° liaison (liaison Lys·Ileu) de la chaîne du zymogène. Cette coupure a deux conséquences: remplacement en position N-terminale du résidu de valine du zymogène par le résidu d'isoleucine de la trypsine et ablation sous la forme d'un hexapeptide ($Val(Asp)_4Lys$) de la séquence N-terminale située en deçà de la liaison coupée[5-7].

Par contre, le mécanisme d'activation par l'entérokinase n'est pas encore définitivement établi. YAMASHINA[8] a bien constaté qu'en traitant le trypsinogène par une préparation purifiée d'entérokinase, il apparaissait, comme pendant l'autoactivation, un résidu N-terminal d'isoleucine dont les proportions augmentaient avec le degré d'activation. Mais, selon toute vraisemblance, l'autoactivation se déclenche

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Abréviations utilisées: DFP, diisopropylfluorophosphate, DP-trypsine, enzyme spécifiquement inhibé par 1 mole du réactif précédent; FDNB, fluorodinitrobenzène; DNP, radical dinitrophényl.

déjà à pH 5.6. Si l'on omet de paralyser l'activité trypsique par un moyen quelconque, cette activation peut jouer un rôle prépondérant, et il devient difficile de savoir ce qui revient réellement à l'entérokinase dans les phénomènes observés. Les expériences de YAMASHINA devraient être refaites en présence d'un inhibiteur spécifique de la trypsine.

Quant à l'activation par l'enzyme du *Penicillium*, rien de précis n'est connu à son sujet. Elle est pourtant intéressante car elle s'effectue à un pH suffisamment acide pour que l'autoactivation et l'autolyse ultérieure de la trypsine formée ne soient pas à craindre.

La souche de *Penicillium* de KUNITZ semblant être perdue, nous nous sommes adressés à un enzyme élaboré par *Aspergillus saitoi*. Obtenu récemment à l'état cristallisé, cet enzyme coupe, comme la trypsine, les liaisons basiques. Son pH optimum est 4.5 environ[9].

TECHNIQUES

Matériel d'étude

Après un certain temps de stockage, le trypsinogène commercial manifeste souvent une activité trypsique très appréciable. Il convient donc d'abord, soit de le purifier par chromatographie, soit de choisir un échantillon faiblement activé. Notre échantillon contenait 2 % de trypsine. Il a été simplement traité 15 min à 0° et pH 7.8 par 5 moles/mole de DFP, dialysé contre HCl 10^{-3} M et lyophilisé. Tous les calculs relatifs à cette substance sont basés sur les valeurs suivantes: $E_{1\,\mathrm{cm}}^{1\%}$ à 280 mμ, 13.9; poids moléculaire, 25,000.

L'enzyme cristallisé d'*Aspergillus saitoi* nous a été aimablement fourni par Dr. F. YOSHIDA. Sa préparation, ses propriétés et sa spécificité ont été récemment décrites[9]. Il hydrolyse rapidement à pH 4.5 le substrat caractéristique de la trypsine, le benzoyl-L-argininamide. Mais il s'en différencie nettement par une spécificité beaucoup plus large.

Réalisation des expériences

Extrémités N-terminales: Le trypsinogène (1 % dans l'acétate d'ammonium 0.05 M pH 4.5) est traité à 0° pendant des temps variables par 10^{-3} mole/mole d'enzyme cristallisé. On dose l'activité trypsique sur le benzoyl-L-arginine éthylester et on détermine les extrémités N-terminales apparues par la technique classique de SANGER. Dans le but d'éviter toute action de la trypsine pendant la condensation avec FDNB, on refroidit à 0°, on amène le pH à 7.8 et on ajoute immédiatement 50 moles/mole de DFP. Puis, on agite avec FDNB en présence d'éthanol et de triméthylamine, d'abord 2 h à 0°, puis 2 h à 20°. Après extraction à l'éther, on acidifie et on extrait plusieurs fois par HCl N, l'éthanol, la méthyléthylcétone et l'éther. On admet que la partie insoluble contient des DNP-protéines et que la partie soluble contient des DNP-peptides. Les 2 fractions sont hydrolysées séparément et les hydrolysats sont étudiés de la manière habituelle.

Extrémités C-terminales: Après avoir traité le trypsinogène par l'enzyme pendant 4 h, on porte à 80° pendant 3 min à pH 2.4 pour arrêter la réaction, on refroidit 30 min dans la glace pour laisser à la trypsine le temps de se renaturer et on dialyse contre HCl 10^{-3} M. Puis, on élimine par 2 lyophilisations successives l'eau et l'acétate d'ammonium du dialysat (D) et du liquide dialysé (L).

Les extrémités C-terminales de (L) sont alors déterminées par la technique à la carboxypeptidase-A et la technique d'hydrazinolyse sous sa forme modifiée[10]. La première technique est utilisée comme suit: Afin d'inhiber la trypsine, on traite le liquide L 15 min à 0° par 20 moles/mole de DFP dans un tampon véronal 0.02 *M* pH 7.8 contenant 0.01 % de Tween 20. On ajoute à 25° 0.03 mole/mole de carboxypeptidase-A préalablement dialysée et traitée par DFP. Les amino acides libérés sont déterminés et dosés par la méthode de LEVY[11].

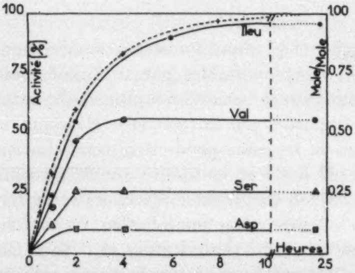
Chromatographie des peptides: Les peptides de (D) sont séparés par passage à travers une colonne de Dowex-50 x 2 dans les conditions précisées dans la légende de la Fig. 3. Une fraction sur quatre est traitée par la ninhydrine en présence de $SnCl_2$[12]. La composition de chaque pic est étudiée en prélevant des échantillons au sommet et sur les branches, en échangeant leurs ions avec ceux de l'acétate d'ammonium, en éliminant ces derniers sous vide et en effectuant des chromatographies sur papier avant et après hydrolyse.

RESULTATS EXPÉRIMENTAUX

Etude de l'activation et extrémités N-terminales

La Fig. 1 indique la cinétique de l'activation et permet de connaître les proportions des résidus N-terminaux au cours de ce processus. On voit que l'activation est assez rapide et qu'elle est "complète", en ce sens que 1 mole de trypsinogène donne naissance dans les conditions de l'essai à une activité contre le benzoylarginine éthylester égale à celle de 1 mole de la trypsine classique. Comme le montre la Fig. 2, la réaction d'activation est du 1° ordre. Nous sommes donc certainement en présence, non pas de l'activation autocatalytique par la trypsine, mais d'une nouvelle activation réalisée par l'enzyme d'*Aspergillus saitoi* agissant de façon autonome.

Au cours de cette activation, 4 amino acides: l'isoleucine, la valine, la sérine et l'acide aspartique (ou l'asparagine) apparaissent en position N-terminale dans la



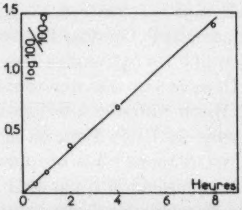


Fig. 1. Activation et résidus N-terminaux des "protéines" du mélange. Les ordonnées de droite indiquent le % d'activité, en prenant pour base 100 l'activité maximum qu'acquiert une même quantité de trypsinogène par activation autocatalytique. Les ordonnées de gauche indiquent le nombre de résidus N-terminaux par mole de trypsinogène en jeu. +, % d'activité vis à vis du benzoyl-L-arginine éthylester. ●, △ et ⊡, respectivement, isoleucine, sérine et acide aspartique apparus en position N-terminale pendant l'activation. ⊙, valine N-terminale formée au cours de l'activation, compte-tenu de la valine N-terminale du trypsinogène (voir le texte).

Fig. 2. Ordre de la réaction d'activation. *a* est l'activité au temps *t* en % de l'activité potentielle totale. 100 représente donc la quantité initiale de trypsinogène et 100—*a*, la quantité restante au temps *t*.

fraction protéique des mélanges. Les proportions d'isoleucine sont considérables. Suivant fidèlement le % d'activation, elles atteignent 1 mole/mole quand l'activation est complète. Pour ce qui concerne la valine, il faut tenir compte du résidu N-terminal que contient le trypsinogène et qui passe progressivement dans la fraction peptidique pendant l'activation (Tableau I). Nous calculons donc la valine réellement formée en retranchant de la valine totale la valine appartenant au trypsinogène restant, elle-même déduite du % d'activation. C'est ainsi par exemple qu'après 2 h, la quantité totale de valine N-terminale déterminée à l'état de dérivé DNP est 0.86 mole/mole alors que le trypsinogène contient 0.97 mole/mole de valine N-terminale et que le % d'activation est 60. La quantité de valine N-terminale apparue en 2 h est:

TABLEAU I

ETUDE PRÉLIMINAIRE DES PEPTIDES FORMÉS AU COURS DE L'ACTIVATION

Durée de l'activation (h)	*Résidus après hydrolyse (mole par mole de trypsinogène en jeu)*			
	DNP-Val	*ε-DNP-Lys*	*Asp*	*Autres résidus*
1	0.2	—	0.8–1.0	0
2	0.8	0.5	2–3	0.2
23.5	0.9	1.0	3–4	1.3

$$0.86 - 0.40 \times 0.97 = 0.47 \text{ mole/mole}$$

La Fig. 1 montre que la formation de valine N-terminale est presqu'aussi rapide au début que celle de l'isoleucine. Puis, elle s'arrête brusquement dès que l'activation est à peu près complète. La quantité maximum est d'environ 0.5–0.6 mole/mole. Les proportions de sérine et d'acide aspartique N-terminaux sont beaucoup plus faibles. Elles ne dépassent pas 0.25 et 0.10 mole/mole, respectivement.

Le Tableau I indique les DNP-amino acides et les amino acides libres formés par hydrolyse de la fraction "DNP-peptides".

Les "autres résidus" mentionnés dans la dernière colonne du Tableau I étant au nombre d'environ 9 et leurs proportions individuelles n'étant jamais supérieures à 0.3 mole/mole, nous pouvons dire que la fraction "DNP-peptides" contient surtout DNP-Val, ε-DNP-Lys et Asp dans le rapport molaire 1:1:3–4. Le peptide principal engendré par l'activation est donc très vraisemblablement le même que celui de l'auto-activation, à savoir: Val(Asp)$_4$Lys. Ce fait va être confirmé un peu plus loin par chromatographie directe des peptides sur Dowex-50.

Etude des extrémités C-terminales

Traitée par la carboxypeptidase-A pendant 15 min, la partie protéique des mélanges (durée de l'incubation: 4 h) engendre de nombreux amino acides parmi lesquels on distingue: l'asparagine (0.6 mole), la sérine (0.5 mole), la tyrosine (0.5 mole), la valine (0.4 mole), la thréonine (0.4 mole), la leucine ou l'isoleucine (0.3 mole). Par hydrazinolyse, cette même fraction fournit: 0.2 mole de sérine et 0.3 mole de glycocolle.

Etude des peptides libérés pendant l'activation

La Fig. 3 reproduit le diagramme obtenu en chromatographiant les peptides selon la technique de MOORE ET STEIN[12]. Ce diagramme met en évidence 7 pics plus ou moins bien différenciés dont la composition et les proportions sont données dans le Tableau II.

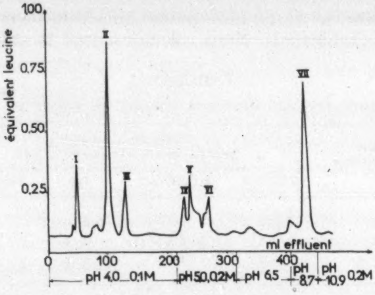


Fig. 3. Chromatographie des peptides sur Dowex-50. Colonne (0.9 × 60 cm) de Dowex-50 × 2 (parties égales de grains à 100–200 et 200–400 mailles). Débit, 4 ml/h. Fractions de 1 ml. Les liquides d'élution sont, dans l'ordre, tampon citrate 0.1 *M* pH 4.0, tampon citrate 0.2 *M* pH 5.0, tampon citrate 0.2 *M* pH 6.5, bicarbonate 0.2 *M* (pH 8.45) et carbonate 0.2 *M* (pH 10.9).

TABLEAU II

ANALYSE DES PICS DE LA Fig. 3

L'équivalent-leucine de chacune des substances est supposé être égal à 1, sauf dans le cas des pics II et III où il est pris égal à 1.75 pour tenir compte de la présence d'un résidu de lysine[13]. Les astériques (*) indiquent que le pic considéré contient plusieurs peptides dont les proportions individuelles sont très faibles.

No. des pics	*Composition*	*Nb de moles pour 1 mole de trypsinogène*
I	Val(Asp)$_3$	0.12
	Ser	0.24
II	Val(Asp)$_4$Lys	0.75
III	Asp·Lys	0.14
IV	Ammoniaque	—
V	*	—
VI	*	—
VII	*	0.90

DISCUSSION

Les résultats obtenus ci-dessus peuvent être commentés comme suit:

1. Le fait que la réaction d'activation se produise à pH 4.5 et qu'elle soit d'ordre 1, prouve que cette réaction n'est pas due à la trypsine. Des expériences-témoins montrent d'ailleurs que le trypsinogène traité à 0° et pH 4.5 par 20 % de trypsine pendant 5 h ne s'active qu'à concurrence de 6 %. Nous nous trouvons donc bien en présence d'un nouveau mode d'activation du trypsinogène dont le mécanisme mérite d'être éclairci.

2. Les deux phénomènes principaux que l'on observe au cours de cette nouvelle activation sont identiques à ceux qui accompagnent l'autoactivation. Un résidu N-terminal d'isoleucine (Fig. 1) et une mole d'hexapeptide Val(Asp)$_4$Lys (Tableau I et Fig. 3) apparaissent par mole de trypsinogène en jeu. Une très bonne corrélation

peut, d'autre part, être établie entre les deux phénomènes précédents et le degré d'activation. En outre, aucun résidu C-terminal n'est engendré en proportions stoechiométriques pendant l'activation. Il semble donc évident que le trypsinogène est activé par l'enzyme d'*Aspergillus saitoi* de la même façon que par la trypsine. Dans l'un et l'autre cas, l'activation est provoquée par la rupture de la 6° liaison (liaison Lys·Ileu) de la chaîne.

3. Néanmoins, l'enzyme d'*Aspergillus saitoi* fait également apparaître dans la fraction protéique des mélanges les résidus N-terminaux suivants: valine (0.56 mole), sérine (0.26 mole) et acide aspartique (0.1 mole). Tous ces résidus sont-ils caractéristiques de l'activation par *Aspergillus saitoi* où se forment ils aussi, par une série de réactions secondaires, au cours de l'autoactivation? Pour le savoir, on traite une solution de trypsinogène à 1 % pendant 15 h à pH 7.8 par 2.3 % de trypsine. L'activation est totale. On trouve dans la partie protéique de l'isoleucine N-terminale (0.95–1.0 mole), de la valine (0.10–0.15 mole), de la sérine (0.25 mole) et de l'acide aspartique (0.10 mole). Seule la valine semble donc caractéristique de la nouvelle activation.

4. Etant donné que la formation de valine N-terminale cesse brusquement au moment où l'activation est complète, on peut supposer que l'enzyme d'*Aspergillus* est capable de rompre une liaison de la valine dans le trypsinogène et qu'il est incapable de rompre cette même liaison dans la trypsine. Une telle hypothèse est confirmée par l'expérience suivante: On traite par l'enzyme une solution à 1 % de DP-trypsine dans des conditions identiques à celles utilisées pour l'activation complète du trypsinogène. La technique ordinaire indique qu'il s'est formé 0.26 mole de sérine N-terminale et 0.1 mole seulement de valine. Nous sommes donc en présence d'un exemple particulièrement frappant de l'influence exercée par la structure tertiaire d'une protéine sur la protéolyse de certaines de ses liaisons. Un autre exemple est fourni, rappelons-le, par la chymotrypsine-π qui autolyse rapidement une liaison Leu·Ser dans sa propre molécule et qui n'attaque pas cette même liaison dans le chymotrypsinogène[14].

5. L'étude réalisée au moyen de la carboxypeptidase-A et de l'hydrazinolyse n'a pas donné de résultats très nets. Elle prouve néanmoins, comme nous l'avons déjà signalé, qu'aucun résidu C-terminal n'apparaît en quantités stoechiométriques pendant l'activation. Mais la nature du résidu C-terminal qui, en toute logique, doit correspondre à la valine N-terminale, n'apparaît pas clairement. La comparaison entre les résultats fournis par les deux techniques suggère néanmoins d'attribuer à l'asparagine une signification particulière. C'est en effet l'amino acide le plus abondant parmi ceux libérés par la carboxypeptidase-A; l'hydrazinolyse, laquelle donne par ailleurs des résultats à peu près négatifs, n'est pas susceptible de le déceler.

En somme, il paraît bien établi que l'enzyme d'*Aspergillus* coupe principalement deux liaisons du trypsinogène: la liaison stratégique Lys·Ileu qui correspond à sa spécificité principale[9] et une autre liaison reliant peut-être un résidu d'asparagine à un résidu de valine. Cette rupture supplémentaire n'est d'ailleurs pas surprenante car, comme toutes les protéinases des microorganismes, l'enzyme d'*Aspergillus* possède une spécificité beaucoup moins étroite que la trypsine[9]. On notera à l'appui de cette proposition que le mélange de peptides illustré par la Fig. 3 contient des quantités notables de Val(Asp)$_3$ et de Asp·Lys. L'enzyme a donc coupé l'hexapeptide Val(Asp)$_4$ Lys au niveau d'une liaison Asp·Asp.

6. Si l'hypothèse d'une rupture supplémentaire est admise, nous sommes conduits à penser que cette rupture n'influe pas sur l'activité estérasique de la trypsine et que l'enzyme d'*Aspergillus* engendre à la fois la trypsine classique et une "neotrypsine" possédant deux chaînes ouvertes. Mais rien de définitif ne peut être dit à ce sujet avant d'avoir séparé et caractérisé les protéines formées pendant l'activation.

7. Signalons pour terminer que l'enzyme d'*Aspergillus saitoi* active également le chymotrypsinogène-A de boeuf. Les courbes de la Fig. 4 montrent que cette activation est plus lente que celle du trypsinogène. Il faut utiliser à la fois une quantité plus grande d'enzyme (rapport molaire enzyme-substrat $2 \cdot 10^{-2}$ au lieu de 10^{-3}) et une température plus élevée (25° au lieu de 0°) pour que les deux activations progressent à un rythme comparable. Dans ces conditions, la ou les chymotrypsines formées sont assez rapidement inactivées. Néanmoins, l'allure de la courbe III de la Fig. 4 suggère que, si aucune inactivation se produisait, l'activité spécifique des mélanges atteindrait vraisemblablement celle de la chymotrypsine-π, c'est à dire la valeur maximum obtenue au cours de l'activation classique par la trypsine.

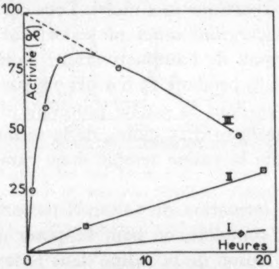


Fig. 4. Activation du chymotrypsinogène-A par l'enzyme d'*Aspergillus saitoi*. L'ordonnée 100 correspond à l'activité spécifique de la chymotrypsine-S. Courbe I: rapport molaire enzyme/substrat: 10^{-3}. Température, 0°. Ces conditions sont identiques à celles utilisées pour l'activation du trypsinogène (Fig. 1). Courbe II: rapport molaire enzyme/substrat: $2 \cdot 10^{-2}$. Température, 0°. Courbe III: rapport molaire enzyme/substrat: $2 \cdot 10^{-2}$. Température, 25°.

RÉSUMÉ

1. Un enzyme protéolytique obtenu à l'état cristallisé à partir d'*Aspergillus saitoi* est capable d'activer le trypsinogène et le chymotrypsinogène-A à pH 4.5. Dans le cas du trypsinogène, l'activation est rapide à 0°. Elle suit une cinétique d'ordre 1 et permet d'atteindre une activité spécifique égale à la valeur maximum obtenue par le processus classique d'autoactivation.

2. Le mécanisme de cette nouvelle activation paraît identique à celui de l'autoactivation. Il comporte comme cette dernière la rupture spécifique de la 6° liaison (liaison Lys·Ileu) de la chaîne du zymogène. Toutefois, par suite de sa spécificité plus large, l'enzyme d'*Aspergillus saitoi* semble capable de couper dans le trypsinogène une autre liaison, sans que l'activité finale en soit diminuée.

BIBLIOGRAPHIE

[1] M. Kunitz, *J. Gen. Physiol.*, 22 (1939) 293.
[2] M. R. McDonald et M. Kunitz, *J. Gen. Physiol.*, 25 (1941) 53.
[3] M. Kunitz, *J. Gen. Physiol.*, 22 (1939) 429.
[4] M. Kunitz, *J. Gen. Physiol.*, 21 (1938) 601.
[5] M. Rovery, C. Fabre et P. Desnuelle, *Biochim. Biophys. Acta*, 9 (1952) 702.
[6] E. W. Davie et H. Neurath, *J. Biol. Chem.*, 212 (1955) 515.
[7] P. Desnuelle et C. Fabre, *Biochim. Biophys. Acta*, 18 (1955) 49.
[8] I. Yamashina, *Acta Chem. Scand.*, 10 (1956) 739.
[9] F. Yoshida et M. Nagasawa, *Bull. Agr. Chem. Soc. Japan*, 20 (1956) 257, 262.
[10] J. H. Bradbury, *Nature*, 178 (1956) 912.
[11] A. L. Levy, *Nature*, 174 (1954) 126.
[12] S. Moore et W. H. Stein, *J. Biol. Chem.*, 176 (1948) 367.
[13] P. Desnuelle et C. Gabeloteau, *Arch. Biochem. Biophys.*, 67 (1957) 475.
[14] M. Rovery, M. Poilroux, A. Yoshida et P. Desnuelle, *Biochim. Biophys. Acta*, 23 (1957) 608.

ON THE METABOLIC STABILITY OF NUCLEIC ACIDS IN MITOTICALLY INACTIVE ADULT TISSUES LABELED DURING EMBRYONIC DEVELOPMENT

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SUMMARY

[8-^{14}C]adenine was administered to pregnant mice in order to incorporate isotope into DNA and RNA of dividing fetal tissue (brain and liver) that subsequently becomes mitotically inactive in the adult animal. The radioactivity in the DNA and RNA purines of these and other tissues from the offspring were determined at periods from nine days to one year after birth. During this period there was no loss of ^{14}C from the DNA purines of brain and liver, whereas the RNA of these tissues did lose radioactivity. These results further confirm the metabolic stability of DNA and indicate that radioactive atoms incorporated into DNA of the liver and brain of embryos may remain fixed for the life of the animal.

INTRODUCTION

The results of many studies in a variety of biological systems have pointed to the metabolic stability of deoxyribonucleic acid (DNA). In bacteria[1,2], in mammalian cells in tissue culture[3], and in the intact animal[4–9], convincing evidence has been obtained that DNA is synthesized only for cell division and, once synthesized, is metabolically inert in both rapidly dividing and in resting cells. The stability of DNA in resting mammalian cells has been emphasized in studies with liver[6,7] in which it was shown that DNA, labeled with an isotope during regeneration following partial hepatectomy, showed no significant loss of activity after regeneration was complete.

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In the work with liver cited above, the stability of nucleic acids was examined over periods up to three months. The present paper extends this type of study to longer periods of observation and to tissues other than liver. For this study, the developing embryo appeared to be the system of choice. In the embryo, there is rapid cell division and synthesis of DNA in all tissues, and it would therefore be expected that if an appropriate precursor were administered, then DNA in all tissues would be extensively labeled. In those tissues in which cells are being renewed following cellular sloughing or expulsion, it would be expected that labeled nucleic acids would be lost at a rate proportional to renewal. However, in other tissues, such as brain and liver, which increase in cell number and mass during embryonic development but have little or no mitotic activity in the mature animal, it would appear that DNA synthesized by the embryo might be retained for the life of original cells and perhaps, in some instances, for the life of the animal. The experiments reported here were designed to study the long-term retention of ^{14}C in the DNA and in the ribonucleic acid (RNA) of mouse tissues labeled during embryonic development by [8-^{14}C]adenine and, at the same time, to see if retention of isotope is such that animals so labeled might be used profitably in future studies of the effects of ^{14}C on somatic cells.

METHODS AND RESULTS

Swiss mice were mated for a 24 h period. Fourteen days later each of five pregnant animals was given a single intraperitoneal injection of [8-^{14}C]adenine at a level of 10 μC/25 g. The animals delivered at the expected time. A group of twenty of the offspring, selected randomly, were sacrificed nine days after birth. After weaning, the remaining offspring (a total of 20 animals) from all litters were pooled, and groups of four to six animals were sacrificed for radioassays at periods from forty days to one year after birth.

The livers, spleens, whole intestines, brains, and (in one group) lungs from each group were pooled separately and homogenized. The homogenate was then extracted three times with cold 5 % trichloroacetic acid (TCA), and the resulting residue was washed with alcohol and ether. For determination of total activity in the crude TCA extracts, measured portions were oxidized, and the resulting $^{14}CO_2$ was counted by a modification of the BERNSTEIN-BALLENTINE method[10]. From the residue, DNA and RNA were separated by the SCHMIDT-THANNHAUSER method, and adenine and guanine from both nucleic acid fractions were isolated on paper chromatograms and assayed for radioactivity by procedures that have been described in detail elsewhere[11]. The results are presented in Table I. In this table are also presented calculations of the ratio of the specific activity of DNA adenine to that of DNA guanine. This ratio provides an additional index of the degree of stability of a nucleic acid. As shown by BENNETT AND KARLSSON[12], if a precursor is used that gives unequal labeling of nucleic acid adenine and guanine, then a change of this ratio with time is a reflection of the breakdown of the nucleic acid, re-equilibration of the adenine and guanine fragments, and reutilization of the equilibrated products. In the particular case of DNA in a

TABLE I

SPECIFIC ACTIVITIES OF ADENINE AND GUANINE FROM DNA AND RNA OF VARIOUS ORGANS OF THE PROGENY OF MICE THAT HAD RECEIVED [8-^{14}C]ADENINE DURING GESTATION

Twenty animals were used in the 9-day group. The other groups each consisted of 4–6 animals. The specific activity values preceded by a "<" sign indicate the presence of some activity in the sample isolated, but so little above background that it could not be assayed accurately.

Organ	*Time after birth (days)*	*Average wt. of organ (mg)*	*Specific activities (counts/sec/µg of purine)*				*Ratio of specific activities* $\frac{\text{DNA adenine}}{\text{DNA guanine}}$
			RNA		*DNA*		
			Ad	*Gu*	*Ad*	*Gu*	
Brain	9	185	0.40	0.23	0.90	0.20	4.5
	40	360	0.14	0.051	0.54	—	—
	71	413	< 0.09	< 0.04	0.53	0.12	4.4
	270	445	0.083	ca. 0.02	0.50	0.11	4.5
	365	452	ca. 0.14	< 0.08	0.48	0.11	4.4
Liver	9	130	0.37	0.17	0.77	0.24	3.2
	40	1520	< 0.02	< 0.02	0.12	ca. 0.04	ca. 3.3
	71	1900	< 0.02	< 0.04	0.093	0.036	2.6
	270	2200	< 0.02	< 0.01	0.058	0.021	2.8
	365	2000	< 0.007	< 0.009	0.050	0.016	3.1
Intestine	9		0.078	0.042	<0.019	—	—
	40		0.011	< 0.02	0.012	< 0.008	
	71		—	—	0.011	—	
	270		< 0.005	< 0.006	0.0088	0.0043	
	365		< 0.006	< 0.002	0.0069	0.0032	
Lung	365	—	< 0.2	< 0.1	0.16	< 0.07	
Spleen*	9	—	0.034	< 0.04	< 0.04	< 0.02	

* There was no measurable radioactivity in spleen purines after nine days.

TABLE II

TOTAL ACTIVITIES PRESENT IN THE VARIOUS FRACTIONS FROM ORGANS OF THE PROGENY OF MICE THAT HAD RECEIVED [8-^{14}C]ADENINE DURING GESTATION

Organ	*Time after birth (days)*	*Total activities ($\mu C \times 10^{-4}$) in TCA extract**	*Specific activities corrected for growth***			
			RNA		*DNA*	
			Ad	*Gu*	*Ad*	*Gu*
Brain	9	7.90	0.40	0.23	0.90	0.20
	40	0.20	0.26	0.10	1.05	—
	71	0.39	—	—	1.14	0.27
	270		0.20	0.048	1.27	0.27
	365		approx. 0.34	—	1.09	0.26
Liver	9	4.28	0.37	0.17	0.77	0.24
	40	1.62			1.39	0.46
	71				1.36	0.53
	270				0.99	0.36
	365				0.77	0.25

* Where no values are given, there was insufficient radioactivity for accurate assay.

** The corrected specific activities at each time period were calculated by multiplying the specific activity at that time by the ratio of the organ weight at that time to the weight at nine days. Where no values are given the specific activities were too low for accurate assay (see Table I).

growing tissue, this ratio obviously may change without breakdown of DNA as a result of turnover of RNA and utilization of RNA breakdown products for synthesis of DNA. The observed ratios (Table I) are invariant for brain; the ratios for liver are more variable, but there is no consistent decline. The greater variability in the liver ratios is probably the result of the lower individual specific activities of the purines, and consequently greater inaccuracy of the assays, as compared to assays of the purines from brain.

Since the tissues were increasing in mass during the earlier part of the experiment, the total activities rather than the specific activities are a measure of the retention of radioactivity in the DNA and RNA purines. On the basis of the observed average weights of each tissue (Table I), the specific activities at the nine-day period were corrected for growth and additional nucleic acid synthesis from inactive or almost inactive precursors, so that the resulting values are a measure of total radioactivity. These corrected specific activities are presented in Table II. No growth corrections were made for intestine because of the very low specific activities (Table I) and the unreliability of a growth correction in tissues known to slough and renew cells at a considerable rate. Table II also contains values for the total activities in the crude TCA extract; it should be noted that the values are given for this crude fraction in absolute microcuries, whereas those for the DNA and RNA purines are specific activities corrected for growth. Because of the crudeness of the samples, the values for the TCA extract are of limited interest. It is perhaps significant that in some tissues

TABLE III

RADIATION DOSAGE PER ORGAN IN THE PROGENY OF MICE THAT HAD RECEIVED [8-^{14}C]ADENINE DURING GESTATION

Tissue	*Time after birth (days)*	*Radiation dosage in mRem/week*		
		RNA	*DNA*	*Total*
Brain	9	95	100	195
	30–365	21	58	79
Liver	9	311	185	496
	30–71	—	26	26
	71–365	—	13	13

TABLE IV

NUMBER OF TRANSMUTATIONS OCCURRING IN DNA OVER A TWO-YEAR LIFESPAN OF PROGENY OF MICE THAT HAD RECEIVED [8-^{14}C]ADENINE DURING GESTATION

Tissue	*Number of transmutations**		
	Per million molecules of DNA	*Per cell*	*Per tissue*
Brain	11.5	66.0	$3.7 \cdot 10^9$
Liver	1.25	8.18	$5.1 \cdot 10^9$
Lung	2.80	18.4	$4.0 \cdot 10^9$

* The molecular weight of DNA[18] was taken as $5 \cdot 10^6$. Literature values for DNA content[9,14] were used in the calculations.

radioactivity was present in soluble form two months after birth of the animal. This radioactivity may come from the turnover of RNA, which did lose some activity during this period. In experiments with adult mice, HENDERSON AND LEPAGE[13] found significant radioactivity in acid-soluble adenine of a number of tissues nine days after administration of [^{14}C]adenine.

Since the results of Table II showed that ^{14}C was retained indefinitely in the DNA of the brain and liver, it was of interest, from the viewpoint of possible biological effects of the resulting radiation, to calculate the radiation dosage resulting from the ^{14}C present in the DNA (and also in the RNA when the activity was significant). Table III presents the results of these calculations in terms of mRem* per week. Table IV presents the number of transmutations theoretically occurring in the various organs over the assumed two-year life span of the animal calculated on the assumption that the DNA would be conserved for the last year of life as it was during the first. In calculating the values of Tables III and IV, pool sizes of DNA and RNA for the various tissues were taken from the literature[9,14] and, in some instances, were for animals other than the Swiss mice used in this experiment. However, the values calculated can be taken as indicating the correct orders of magnitude.

DISCUSSION

The data of Tables I and II indicate clearly that the ^{14}C present in the DNA purines of liver and brain of the nine-day old animal is retained without loss for the next twelve months—the limit of these experiments. During the first two months of this period, these tissues were increasing in mass and thereafter remained essentially constant in weight; thus, the DNA was conserved in both dividing and in resting cells. Earlier studies have shown the retention of ^{32}P in the liver of weanling rats[8] and of ^{14}C and ^{15}N in adult liver[6,7]; the studies with ^{32}P were carried out for periods up to ten days and those with ^{14}C and ^{15}N for periods up to three months. From the data of the present experiments, which covered half of the life span of the mouse, it would appear that the isotope incorporated into DNA of the brain or liver may be retained for the life of the animal.

Evidence for the stability of DNA in these tissues lies both in the retention of total activity when corrections are made for increases in mass of tissue (Table II) and in the invariance of the ratio of the specific activity of adenine to that of guanine (Table I). Although these results do not preclude the possibility that DNA may be in equilibrium with, or exchange molecules with soluble precursors, they strongly suggest that this does not happen. If there is any such exchange or equilibrium, then any breakdown products of DNA would have to be completely reutilized for synthesis of DNA and in such a manner that the magnitude of the Ad:Gu specific activity ratio would be unchanged. This is unlikely, since the pool size of acid-soluble adenine derivatives of liver and other tissues that have been studied is much greater than the pool size of soluble guanine derivatives[9].

The biochemical inertness of DNA and the parallel between DNA synthesis and cell division now appear to be generally accepted. Nevertheless, PELC has recently obtained evidence that in the seminal vesicles of the mouse there is synthesis of DNA that is unrelated to cell division and has cited evidence indicating that the DNA of liver is being renewed at a rate of about 1 % per day[15–17]. The data of Table II provide no evidence of any significant renewal of DNA in brain or liver over a one-year period. However, in view of the heterogeneity of DNA, these data cannot be interpreted as indicating anything more than that certain DNA fractions that were labeled in the embryo are conserved completely. There could well be other DNA fractions that were not labeled under these conditions and that are renewed in the adult animal.

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* mRem = milliroentgen-equivalent-man, defined as that quantity of any type of radiation which, when absorbed by man, produces an effect equivalent to the absorption of 0.001 R of X- or γ-radiation.

The behavior of RNA was different from that of DNA. In the liver, there was such an increase in weight between nine days and forty days (Table I) that the specific activities of the RNA purines would be lowered by new synthesis alone to very low values. The observed specific activities after nine days were, in fact, too low for accurate assay, and therefore the data do not permit an estimate of the degree of stability of RNA. However, although a quantitative comparison cannot be made, the specific activities of the RNA purines and DNA purines were of the same order at the nine-day period, and the fact that the specific activities of the RNA purines were consistenly lower than those of the DNA purines at the later periods does indicate some loss of activity from RNA. This finding is consistent with those from studies of regenerating liver and the liver of young animals which showed extensive loss of label from RNA without significant loss from DNA[7,8]. In contrast to the liver, the increase in the mass of the brain was very small, and activity could be measured in the RNA purines at all periods of the experiment. The loss of activity from brain RNA appears to be slow and most of the loss occurred between nine and forty days. In considering these results, it should be borne in mind that RNA, like DNA, is a heterogenous material, and it is therefore possible that the RNA fractions that are being rapidly renewed have already lost most of their activity by the time the first measurement was made nine days after the animals were born. If this were so, then the labeled RNA that was studied during the experiment would represent those RNA fractions that were being renewed at a very slow rate.

Among the other tissues studied, intestine and spleen rapidly lost radioactivity from the nucleic acids as is to be expected of tissues of organs that are dividing rapidly and expelling or sloughing cells in order to maintain equilibrium compatible with the host. As compared with liver and brain, the nucleic acids of the intestine and spleen contained little measurable activity at nine days; this fact suggests that most of the activity initially incorporated by these actively dividing tissues had already been lost by this time. The radioactivity present in the intestine at nine days declined slowly; it is probable that his activity is present in the DNA of muscle, which has been shown to have essentially no turnover[15,16]. The lungs were assayed only at the twelve-month period and, hence, retention of isotope could not be determined; however, the specific activity of DNA adenine of lung at this period was intermediate between those of liver and brain.

The retention of isotope in liver and brain is of interest for studies on possible radiation damage, particularly for study of the long-term effects of ^{14}C, localized in the DNA, on somatic cells. The calculations of Table III show that, as a result of the ^{14}C taken up by the embryo, the nucleic acids of the liver and brain are exposed for a period extending from nine days to thirty days after birth of the animal to radiation in excess of the 100 mRem/week recommended by the U.S. Atomic Energy Commission as the maximum permissible level for continuous exposure. After thirty days, the radiation has dropped below this level as a result both of loss of ^{14}C from RNA and increase in tissue mass. It should be noted that the suggested safe radiation level of 100 mRem/week is for total body radiation and hence may not apply to the present situation, because the bulk of the ^{14}C is present as an integral part of the DNA, a site presumed to be particularly sensitive to damage by radiation. Of equal interest with regard to biological effects is the number of transmutations occurring in the DNA (Table IV). The number of transmutations per million molecules of DNA is small; however, when calculations are made on a per cell or per tissue basis, it is apparent that over the life span of the animal the number of transmutations occurring in liver, brain, and lung is appreciable. Since the β-radiation originates in, and the transmutations occur in, the genic chemicals, it is possible that ^{14}C thus incorporated and retained might produce effects on somatic cells that could be detected.

A long-term study is underway to evaluate any such effects of ^{14}C incorporated by the intact animal under these extreme experimental conditions—intraperitoneal administration to pregnant animals of a specific labeled precursor of nucleic acids—in which incorporation and retention of isotope in DNA of certain tissues are maximal.

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REFERENCES

[1] A. D. Hershey, *J. Gen. Physiol.*, 38 (1954) 145.
[2] L. Siminovitch and A. F. Graham, *Can. J. Microbiol.*, 2 (1956) 585.
[3] G. M. Healy, L. Siminovitch, R. C. Parker and A. F. Graham, *Biochim. Biophys. Acta*, 20 (1956) 425.
[4] L. Revesz, A. Forssberg and G. Klein, *J. Natl. Cancer Inst.*, 17 (1956) 37.
[5] J. F. Scott and E. B. Taft, *Biochim. Biophys. Acta*, 28 (1958) 45.
[6] J. R. Fresco, A. Bendich and P. J. Russell, Jr., *Federation Proc.*, 14 (1955) 214.
[7] L. I. Hecht and V. R. Potter, *Cancer Research*, 16 (1956) 988.
[8] H. K. Kihara, M. Amano and A. Sibatani, *Biochim. Biophys. Acta*, 21 (1956) 489.
[9] L. L. Bennett, Jr., H. E. Skipper, L. Simpson, G. P. Wheeler and W. S. Wilcox, *Cancer Research*, 20 (1960) 62.
[10] L. Simpson, *Intern. J. Appl. Radiation and Isotopes*, 3 (1958) 172.
[11] L. L. Bennett, Jr., H. E. Skipper, C. C. Stock and C. P. Rhoads, *Cancer Research*, 15 (1955) 485.
[12] E. L. Bennett and H. Karlsson, *J. Biol. Chem.*, 229 (1957) 39.
[13] J. F. Henderson and G. A. Le Page, *Cancer Research*, 19 (1959) 67.
[14] I. Leslie, in E. Chargaff and J. N. Davidson, *The Nucleic Acids*, Vol. II, Academic Press, Inc., New York, 1955, p. 1.
[15] S. R. Pelc, *Exptl. Cell Research*, 14 (1958) 301.
[16] S. R. Pelc and P. B. Gahan, *Nature*, 183 (1959) 335.
[17] S. R. Pelc, *Lab. Invest.*, 8 (1959) 225.
[18] D. O. Jordan, in E. Chargaff and J. N. Davidson, *The Nucleic Acids*, Vol. I, Academic Press, Inc., New York, 1955, p. 447.

THE NUCLEOTIDE COMPOSITION OF RIBONUCLEIC ACIDS FROM SUBCELLULAR COMPONENTS OF YEAST, *ESCHERICHIA COLI* AND RAT LIVER, WITH SPECIAL REFERENCE TO THE OCCURRENCE OF PSEUDOURIDYLIC ACID IN SOLUBLE RIBONUCLEIC ACID

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SUMMARY

The analyses of the nucleotide composition of RNA's derived from various subcellular fractions of yeast and *Escherichia coli* B, revealed that the RNA found in the supernatant fraction has a unique nucleotide composition as compared with the RNA from the particle fractions. The supernatant RNA is characterized by the presence of a considerable amount of pseudouridylic acid and by higher cytidylic acid and lower adenylic and uridylic acid contents. Predominant localization of pseudouridylic acid in the RNA of the "pH 5 enzyme fraction" of rat liver was also observed.

The relation between the ability to bind [^{14}C]leucine and the content of pseudouridylic acid in RNA's prepared from large granules, small granules, and some subfractions of the 105,000 × *g* supernatant of yeast cells has also been investigated. The RNA prepared from the supernatant fraction is the only active RNA, those prepared from particle fractions failing to incorporate [^{14}C]leucine. In five preparations of RNA derived from the acid-fractionated supernatant subfractions and the unfractionated supernatant, it was found that the ability to incorporate [^{14}C]leucine into different RNA's is directly proportional to their pseudouridylic acid content. These data suggest that this nucleotide is characteristic of the RNA which incorporates amino acids (at least leucine).

INTRODUCTION

It is well known that the RNA in the cells is not homogeneous and shows variations in the nucleotide composition[1–4] and the turnover rate[4–7], which depend on the intracellular location. Such structural and metabolic heterogeneity of cellular RNA's would underlie the heterogeneity of their function. There has been much speculation about the involvement of microsomal RNA in the determination of the specificity of proteins. However, one of the most remarkable and also definite functions is that of the RNA found in the supernatant fraction. It has been reported[8] that RNA

Abbreviations used: RNA, ribonucleic acid; ATP, adenosine triphosphate; Tris, tris (hydroxymethyl) aminomethane.

in the supernatant fraction (soluble RNA) binds amino acids in the presence of ATP and amino acid-activating enzymes, and that amino acids so bound are then transferred to the microsomal ribonucleoprotein particles. The amino acid–RNA complex formed is thus considered to be an intermediate of protein synthesis[8]. As a basis for this unique function of the active RNA there should be a specific molecular structure. It is now generally recognized from the work of HECHT *et al.*[9] and others[10, 11] that the active RNA possesses the specific terminal group (–pCpCpA), the existence of which is required for the attachment of amino acids. It is not, however, likely that the difference between active and inactive RNA's resides only in the presence or absence of the specific terminal group. For a full understanding of the specific function of the active RNA we require a complete knowledge, not only at the terminal group, but also of the primary and secondary structure of the molecule.

The present paper reports that RNA, in the 105,000 × *g* supernatant of yeast and *E. coli* homogenates, as well as in the "pH 5 enzyme fraction" of the rat liver supernatant, contains a considerable amount of pseudouridylic acid. Because this special nucleotide was not found to any significant extent in RNA's of particle fractions, its presence may be, in addition to the specific terminal group, a feature of the active molecule. In support of this idea it has been demonstrated that the ability to incorporate [^{14}C]leucine into different RNA's prepared from the particle fractions, as well as from some subfractions of the 105,000 × *g* supernatant, is directly proportional to their content of pseudouridylic acid.

Preliminary accounts of this work have already been published[12, 13].

EXPERIMENTAL

Organisms

Yeast cells (*Saccharomyces cerevisiae*, Strain Kaneka) were obtained from Drs. S. SIRAI and M. ASAI of the Kanekafuchi Kagaku Co. Ltd., Takasago. *E. coli* B(H) was provided by Dr. R. OKAZAKI of our institute. Rats of an unspecified strain, weighing about 200 g, were used for the preparation of liver fractions.

Culture conditions

For the nucleotide composition analyses of RNA contained in the whole cells as well as in various subcellular fractions, yeast cells were inoculated in the synthetic medium of HALVORSON AND SPIEGELMAN[14] in a 5-l Erlenmeyer flask. Anaerobic conditions were obtained by filling the flask with the medium and sealing it with a rubber stopper connected to a 50 ml-flask containing 5 % pyrogallol in 4 *N* KOH. The cultivation was carried out at 30° under continuous magnetic stirring. After about 14 h (late log phase) cells were collected and washed twice with the medium of CHAO AND SCHACHMAN[15] (CS-medium). For the preparation of RNA or enzymes for the amino acid binding test *in vitro*, late log phase cells grown under simple standing culture at 30° were used.

E. coli was grown in 6 l Tris–glucose synthetic medium[16] in three 5-l Erlenmeyer flasks at 37° in a mechanical shaker. In some experiments, glucose was substituted by lactose. The cells of late log phase were collected, and washed with a cold 0.001 *M* Tris buffer (pH 7.0) containing 0.001 *M* $MgCl_2$ (see ref. 17).

Fractionation of subcellular components

For the RNA nucleotide composition analyses, 5 g wet yeast cells were ground in a hand mortar for 20 min with the occasional addition of quartz sand. About 15 g of sand were required for the complete disruption of the cells during the period indicated. Homogenate was then suspended with a mechanical stirrer in 30 ml of CS-medium. The suspension was centrifuged at 2,240 × *g* for 5 min. The sediment was washed 3 times with CS-medium and designated as the cell wall fraction. The washings were combined with the first supernatant and centrifuged at 10,000 × g for 20 min in a Servall SS-1 centrifuge. The large granule fraction (LG) was sedimented. The volume of the 10,000 × *g* supernatant was adjusted to 150 ml with CS-medium and centrifuged in Spinco L ultracentrifuge (Rotor No. 40) at 105,000 × *g* for 90 min. The clear supernatant was carefully pipetted out, leaving about 1 cm volume from the bottom of the tube (supernatant fraction (Sp)). The yellowish, transparent pellet firmly packed at the bottom was designated as the small granule fraction (SG). In some experiments the Sp fraction was obtained directly by centrifuging the first 2,240 × *g* supernatant at 105,000 × *g* for 90 min.

For the preparation of RNA the subcellular components were obtained from about 50 g wet cells by essentially the same cell fractionation technique as that described above. When the subfraction of the 105,000 × *g* supernatant was prepared, the pH of the supernatant fraction was adjusted to 5.0 or 5.3 with 1 *N* acetic acid in the cold. The turbid solution was immediately centrifuged at 15,000 × *g* for 10 min in a Servall SS-1 centrifuge, yielding the supernatant and the precipitate at each pH. The precipitate was suspended in 50 ml CS-medium and the pH was adjusted to 6.8 with 1 *N* KOH. The pH of the acid-supernatant was also brought to 6.8 with KOH.

The general procedure of fractionating *E. coli* cells was the same as in the case of yeast cells, except the use of Tris–Mg^{++} suspending medium[7]. The first low speed sediment was discarded, because of its negligible content in RNA.

Fractionation of rat liver components was conducted by the method of KELLER AND ZAMECNIK[18].

All operations were done at 0–2°.

Nucleotide composition analyses of RNA

The RNA nucleotide composition of each subcellular fraction was determined by Dowex-1-formate ion exchange chromatography after 0.5 *N* KOH hydrolysis (18 h at 38°) as described previously[4]. For the analyse of the composition of purified RNA, RNA was treated with 1 *N* KOH at 37° for 24 h, to insure more complete hydrolysis. The individual mononucleotides were separated on a 0.6 cm × 28 cm Dowex-1-formate column[4].

For the millimolar extinction coefficient of pseudouridylic acid, 8.3 at 260 mμ at acidic condition[19] was adopted.

RNA preparations*

An equal volume of 90% commercial phenol[20] was added to each subcellular fraction described above and stirred for 1 h at room temperature (about 23°) with a mechanical stirrer. The resulting emulsion was centrifuged in the cold at 16,300 × g (Lourdes Centrifuge, Volume rotor) for 30 min. The aqueous layer was pipetted out. The remainder was mixed with an appropriate volume of cold water and centrifuged again. The combined aqueous layer was made up to 2 % with respect to potassium acetate. 2 volumes of cold ethanol were then added and the content was kept at -5° for 18 h. The precipitate was collected by centrifugation and was dissolved in 20 to 50 ml of water. Insoluble matter was centrifuged down at 105,000 × g for 30 min. The RNA was precipitated again by adding 2 volumes of ethanol and 2 % potassium acetate. It was dissolved in water and dialysed for 4 h against cold water. After dialysis, it was again centrifuged at 20,000 × g for 20 min. The clear solution was then frozen and lyophilized. Dried samples were kept in a desiccator in a cold room before use.

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* The RNA preparations, especially those of the supernatant fraction, are contaminated with polysaccharides. We have some evidence which shows that these polysaccharides do not alter the conclusions presented in this paper. Isolated polysaccharides from this RNA preparation have no effect on the incorporation of [^{14}C]leucine into the RNA contained in the enzyme preparation. The purified RNA free from polysaccharides has a specific activity similar to that of the crude RNA.

Preparation of cell extract as the enzymes for the activity test of RNA

5 g of wet yeast cells, washed with CS-medium, were ground with quartz sand for 20 min. The homogenate was suspended in 15 ml of CS-medium and centrifuged at 2,000 rev./min for 3 min to remove sand. The supernatant was again centrifuged at 105,000 × g for 90 min. The fat layer at the top of the tube was removed and the upper 3/4 of the supernatant was pipetted out and dialysed against 0.003 *M* Tris buffer at pH 6.8. After dialysis for 18 h, the pH of the content was brought to 7.6 by careful addition of saturated potassium bicarbonate. All operations were done at 0–2°. The preparation usually contained about 6 mg proteins and 0.35 mg RNA/ml.

Test of the ability of RNA to combine L-*leucine*

0.2 ml of RNA dissolved in 0.2 *M* Tris buffer (pH 7.6) was shaken at 30° for 5 to 15 min with 0.3 ml of the dialyzed 105,000 × g supernatant, 1 µmole ATP, 1 µmole $MgCl_2$, 13.3 µmoles Tris buffer, pH 7.6, and 0.41 µmole (0.25 µC) L-[^{14}C]-leucine in a total volume of 1 ml. Duplicate or triplicate flasks were usually run. In some experiments, 0.05 µmole cytidine triphosphate was added without effect. Variation in the amount of ATP from 0.5 µmole to 10 µmoles, of the enzymes from 0.2 ml to 0.5 ml, of $MgCl_2$ from 1 µmole to 3.3 µmoles and of Tris buffer from 13.3 µmoles to 50 µmoles, did not change the incorporation of [^{14}C]leucine into RNA. After incubation, the flasks were chilled and 8 ml of 0.4 *N* $HClO_4$, plus 2.0 ml of carrier proteins, were added. The precipitate was washed 4 times with cold 0.2 *N* $HClO_4$, twice with cold ethanol, once with acetone, twice with ether, and dried. The powder was suspended in acetone and deposited quantitatively on Whatman No. 1 filter paper with the aid of a Tracerlab E8A filtration apparatus. The activity of samples was counted in a 2π gasflow counter, and corrected for self-absorption. Duplicate blanks, without added RNA, were always run in parallel. The total activity of added RNA was estimated as the difference between the total activity of experimental series and the total activity of the blank. Similarly the amount of added RNA is known by the difference between the RNA contained in the sample of the experimental series and that contained in the blank. For the determination of RNA, u.v. absorption at 260 mµ was estimated on a hot 10 % $HClO_4$ extract (70°, 20 min) of the sample which had been used for counting.

The L-[^{14}C]leucine used in this work was part of an allocation of it made by The Radiochemical Centre, Amersham, England to The Japan Isotope Association.

RESULTS AND DISCUSSION

Occurrence of pseudouridylic acid in RNA of the supernatant fraction of yeast, E. coli, *and rat liver cells*

In Table I, column 1, are shown the nucleotide compositions of RNA's of different subcellular components of yeast cells. Because no special effort was made in the present study to identify the cells in the fractions used, the fractions collected at the centrifugal field of 10,000 × *g* (20 min), and that at the field of 105,000 × *g* (90 min) were provisionally named the "large granule (LG) fraction", the "small granule (SG) fraction", and the "supernatant (Sp) fraction", respectively. The SG fraction includes the bulk of ribonucleoprotein particles (80 S particles of CHAO AND SCHACHMAN[15]) of the yeast cells. In any event, it is clear from the table that the RNA's from the LG and SG fractions were very similar, whereas that of the RNA of the supernatant fraction was significantly different. The supernatant RNA, as compared with the RNA of the particle fractions, is characterized by a much higher content of cytidylic acid and a lower content of adenylic and uridylic acid.

In addition to the distinct composition of the four nucleotides which commonly occur in SpRNA, the chromatogram of these RNA nucleotides indicated the presence

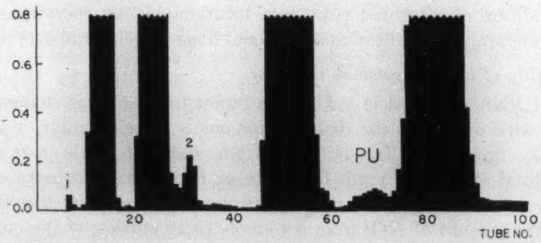

Fig. 1a.

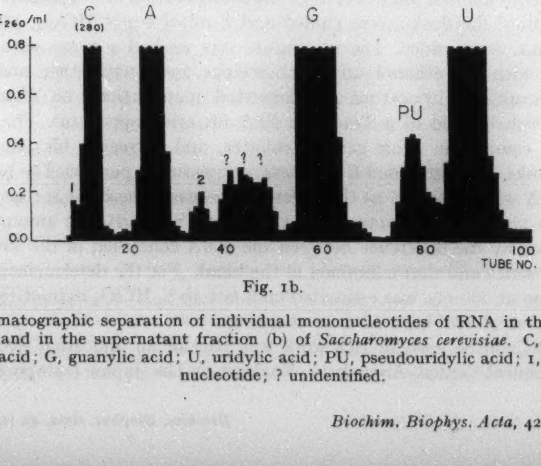

Fig. 1b.

Fig. 1. Chromatographic separation of individual mononucleotides of RNA in the small granule fraction (a) and in the supernatant fraction (b) of *Saccharomyces cerevisiae*. C, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid; PU, pseudouridylic acid; 1,2, probably not nucleotide; ? unidentified.

of a small but definite peak just before that of uridylic acid*. The contents of the tubes of the main part of this peak were pooled and lyophilized to remove formic acid. The u.v. absorption spectra taken at different pH's agreed closely with those reported by DAVIS AND ALLEN[19] for 5-ribosyluracil nucleotide[26, 27]. We therefore tentatively assumed this to be the same nucleotide and, the name "pseudouridylic acid" proposed by COHN[27] for this substance has been adopted throughout the present paper. Our sample showed $\lambda_{\max}$ 262 mμ, $\lambda_{\min}$ 233 mμ, 260/280 ratio = 0.43 in 0.1 N HCl, and $\lambda_{\max}$ 285 mμ, $\lambda_{\min}$ 245 mμ, 260/280 ratio = 2.0 in 0.1 N KOH.

Fig. 1b shows a chromatographic pattern of RNA mononucleotides from the Sp fraction, indicating the presence of a considerable amount of pseudouridylic acid. The peak corresponding to this nucleotide is either absent or insignificantly small in the chromatograms of the other cellular fractions (*cf.* Fig. 1a). A small amount of this nucleotide detectable in the RNA's of particle fractions would possibly be due to the contamination of the supernatant fraction, because none of the fractions obtained were thoroughly washed to remove traces of the supernatant fluid. However, the possibility of the presence of a very small amount of this substance in RNA's of the particle fractions remains to be examined.

The essential results obtained in the yeast cell fractions were also confirmed in the nucleotide composition analyses of RNA's of *E. coli* subcellular fractions**. The data shown in Table I, column 2, indicated the following characteristics of the SpRNA compared with RNA's from the particle fractions: (a) a higher cytidylic acid content, (b) lower adenylic acid and uridylic acid contents, and (c) a predominant localization of pseudouridylic acid.

Following the confirmation of the distinct composition of SpRNA both of yeast and *E. coli* cells, our interest was focused to see whether the SpRNA of mammalian tissues also has the same features. In this study, the nucleotide composition of microsomal RNA and that of the RNA in the "pH 5 enzyme fraction" obtained from the 105,000 × *g* supernatant of rat liver was examined. Predominant localization of pseudouridylic acid in the latter RNA was evidenced. These results accord well with those reported by DUNN[21], who used the same materials. Although a lower content of uridylic acid in the RNA of the pH 5 enzyme fraction was found, no characteristic differences in adenylic acid and cytidylic acid contents were detected (Table I, column 3). It should, however, be pointed out that the composition of the RNA of the pH 5 enzyme fraction is not very different from that of SpRNA of microbial

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* Throughout this paper, we have not discussed the other additional ribonucleotide components in RNA (cf. 21–25), because we have not yet made detailed analyses of these. Examination, however, of the chromatographic patterns suggest that they also are concentrated in SpRNA.

** A comparison of the nucleotide composition of RNA's in *E. coli* cells grown in glucose with those grown in lactose media revealed another aspect of SpRNA. As seen in Table I, Column 2, the composition of RNA's from the LG and SG fractions of glucose- and lactose-grown cells were very similar. A clear difference was unexpectedly obtained in the composition of the SpRNA. The cells which are synthesizing β-galactosidase (in lactose) contained more adenylic acid and less cytidylic acid in the SpRNA, compared with those not forming the enzyme. The behavior of the SpRNA during β-galactosidase formation was closely similar to that found in chloroplast formation in *Euglena gracilis*[28, 29] and, although the analyses were made only on the total RNA, higher adenylic and lower cytidylic acid contents of RNA of the adaptive chloroplast formation were reported. It is not known whether the changes in the nucleotide composition of SpRNA found in our work is in some manner connected with the general mechanism of the inductive synthesis of new proteins, or is related to the determination of the specificity of the particular protein molecule in question.

TABLE I

NUCLEOTIDE COMPOSITION OF RNA'S OF SUBCELLULAR COMPONENTS OF *Saccharomyces cerevisiae*, *Escherichia coli* B(H) AND RAT LIVER

Fraction	*Distribution of RNA in cell %*	*No. of determination*	*Adenylic*	*Guanylic*	*Cytidylic*	*Uridylic*	*Pseudouridylic*
Saccharomyces cerevisiae							
Whole cells	100	3	10.0[*] (25.6)[**]	11.0 (28.2)	7.6 (19.5)	10.4 (26.7)	< 0.3 (—)
Cell wall	6.4	—	—	—	—	—	—
Large granules	7.7	3	10.0 (26.4)	10.2 (26.9)	7.5 (19.8)	10.2 (26.9)	< 0.1 (—)
Small granules	69.0	4	10.0 (25.9)	10.7 (27.7)	7.3 (18.9)	10.6 (27.4)	< 0.3 (—)
Supernatant	14.3	4	10.0 (21.8)	12.5 (27.2)	11.9 (25.9)	10.1 (22.0)	1.4 (3.05)
Escherichia coli, B(H)							
Whole cells, G[***]	100	3	10.0 (23.2)	13.8 (32.4)	9.8 (22.8)	9.4 (20.9)	< 0.1 (—)
L[§]		2	10.0 (23.9)	13.4 (32.0)	9.3 (22.2)	9.1 (21.8)	< 0.1 (—)
Large granules, G	16.6	2	10.0 (24.6)	12.6 (31.0)	9.4 (23.1)	8.7 (21.4)	< 0.1 (—)
L		3	10.0 (24.3)	12.6 (30.6)	9.1 (22.1)	9.5 (23.0)	< 0.1 (—)
Small granules, G	60.0	3	10.0 (24.2)	13.1 (31.9)	8.9 (21.6)	9.3 (22.5)	< 0.1 (—)
L		3	10.0 (24.4)	13.3 (32.4)	8.6 (21.0)	9.2 (22.4)	< 0.1 (—)
Supernatant, G	23.4	2	10.0 (20.0)	15.5 (31.0)	14.3 (28.6)	9.6 (19.2)	0.61 (1.22)
L		2	10.0 (22.0)	14.5 (31.9)	11.7 (25.8)	8.8 (19.4)	0.41 (0.9)
Rat liver							
Microsomes	—	2	10.0 (18.6)	17.2 (31.9)	15.6 (29.0)	11.0 (20.4)	< 0.3 (—)
pH 5 enzyme fraction	—	2	10.0 (19.7)	14.8 (29.2)	14.6 (28.8)	9.2 (18.2)	2.0 (3.95)

[*] Molar ratio, Adenylic acid = 10.0.

[**] Moles/100 moles nucleotides. In this paper, this value is in all cases only tentative, because the additional nucleotides other than pseudouridylic acid were not taken into account.

[***] G = glucose grown cells.

[§] L = lactose grown cells.

cells. On the other hand, the RNA's of the particle fractions were quite different from each other.

The relation of pseudouridylic acid content in RNA's to the ability to link leucine in vitro

It has been shown by HECHT *et al.*[9] and PREISS *et al.*[11] that the ability of RNA to link amino acids can be estimated by measuring the incorporation of labeled amino acids into the added RNA in the presence of ATP and the cell extract containing amino acid-activating enzymes. We have constructed the similar system described above in the section entitled EXPERIMENTAL. In our system, the labeling of the added RNA with [^{14}C]leucine is completed within 10 min and the total count in the RNA is nearly proportional to the amount of RNA added in the 10 to 15 min incubation at 30°. The presence of enzyme is essential. The omission of ATP reduced the incorporation to about one third. When there is a considerable incorporation without the addition of ATP, this may be due to the presence of ATP in the enzyme preparation, which was used undialyzed. The addition of ribonuclease (200 μg/ml) completely abolished the activity. In these experiments the RNA prepared from pH 5.0 supernatant of the 105,000 × *g* supernatant fraction was used as the amino acid acceptor.

The fact that the labeling is almost entirely in the RNA was established by recounting the samples after treatment with hot $HClO_4$ or ribonuclease.

In the first place an examination was made of the incorporation of [^{14}C]leucine into RNA's prepared from different subcellular fractions and also of their nucleotide composition. As Fig. 2 shows and as HOAGLAND *et al.*[8], and HECHT *et al.*[9] also showed in their experiments on animal cells, only the RNA prepared from the supernatant fraction was active as the acceptor of leucine. On the other hand, RNA's from the large granule and small granule fractions were entirely lacking in ability to incorporate

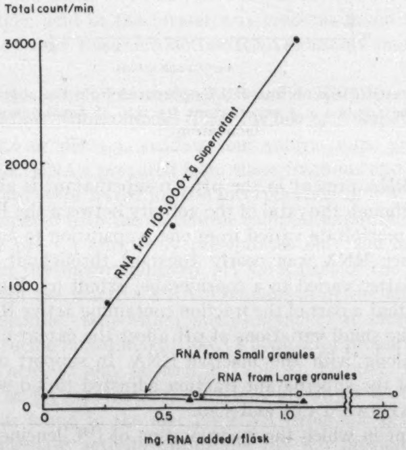

Fig. 2. Incorporation of L-[^{14}C]leucine into RNA's prepared from different subcellular fractions of *Saccharomyces cerevisiae*. 10 min incubation.

[^{14}C]leucine. Nucleotide composition analyses of these RNA preparations revealed that the active RNA contains about 4.5 % of pseudouridylic acid, whereas RNA's from the large granule and small granule fractions have only a trace of this nucleotide. The result is in good accord with the analyses done on the total RNA contained in each fraction.

It was soon found, however, that the RNA in the supernatant was not homogeneous. The specific activity of RNA prepared from the pH 5.0 supernatant is higher than that prepared from the pH 5.0 precipitate of the 105,000 × *g* supernatant (Fig. 3). It is therefore not possible to decide whether pseudouridylic acid is in the active RNA. This situation makes it necessary to correlate the pseudouridylic acid content and the amino acid-binding activity in RNA's prepared from the different subfractions of the supernatant. The quantitative estimation in one case indicated that the amount of RNA present in the pH 5.0 supernatant is about equal to that in the precipitate. Although the ratio of the activity between the RNA of the pH 5.0 supernatant and the precipitate varied from one preparation to another, the specific activity of the former RNA was nearly constant throughout the preparations, whereas that of the latter varied to a considerable extent (*cf.* Fig. 3 and Table II). This probably means that a part of the fraction containing active RNA is precipitated at pH 5.0 and that the small variations of pH affect the extent of the precipitation of the active RNA along with the inactive RNA. In support of this idea it was found that the pH of the supernatant fraction adjusted to 5.0 was shifted, during the centrifugation, to between 5.05 and 5.20.

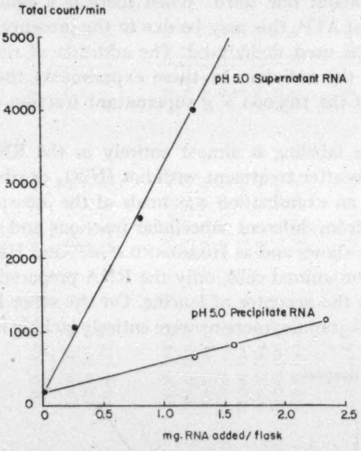

Fig. 3. Incorporation of L-[^{14}C]leucine into RNA's prepared from the supernatant and the precipitate at pH 5.0 of the 105,000 × *g* supernatant fraction of *Saccharomyces cerevisiae*. 15 min incubation.

In one experiment in which the incorporation of [^{14}C]leucine into RNA's from pH 5.0 supernatant and precipitate was measured, the two RNA preparations were analyzed for their nucleotide composition. As Table II, Expt. 1 shows, the pH 5.0

TABLE II

INCORPORATION OF L-[^{14}C]LEUCINE INTO RNAS PREPARED FROM SEVERAL SUBFRACTIONS OF THE 105,000 × *g* SUPERNATANT, COMPARED WITH THE NUCLEOTIDE COMPOSITION OF THESE RNA PREPARATIONS OF *Saccharomyces cerevisiae*, STRAIN KANEKA

0.5 to 1.5 mg RNA per flask were incubated for 15 min under the conditions described in EXPERIMENTAL

Source of RNA	*Nucleotide composition, moles/100 moles nucleotides*						*Amount of L-leucine linked to RNA, mµmole/mg RNA*	
	Adenylic	*Guanylic*	*Cytidylic*	*Uridylic (1)*	*Pseudo-uridylic (2)*	*(1) + (2)*	*(3)*	*(3)/(2)*
Expt. 1								
pH 5.0 supernatant	21.2	27.2	25.2	21.8	4.66	26.46	1.68	0.366
pH 5.0 precipitate	23.9	27.4	22.0	24.4	2.38	26.78	0.92	0.367
Expt. 2								
Unfractionated supernatant	21.1	28.3	24.4	22.1	4.45	27.55	1.68	0.368
pH 5.0 supernatant	20.8	27.2	24.3	22.2	5.57	27.77	2.01	0.361
pH 5.3 supernatant	20.7	28.0	24.6	20.9	5.74	26.64	2.03	0.354
pH 5.0 precipitate	24.8	28.9	19.5	25.5	1.32	26.82	0.47	0.356
pH 5.3 precipitate	25.5	30.3	17.5	25.9	< 0.50	(26.40)	0.00	—
Small granules	25.6	29.0	18.6	26.8	< 0.10	(26.90)	0.00	—

supernatant RNA was about twice as active as the precipitate RNA and the content of the pseudouridylic acid in the former was twice as much as that in the latter. It was also noticed that the more active RNA contains more cytidylic acid and less uridylic acid.

To evaluate this result more closely, several RNA preparations were made from the supernatant subfractions. The 105,000 × *g* supernatant fraction was adjusted to pH 5.0 or pH 5.3, yielding four subfractions, precipitates and supernatants at each pH. RNA's prepared from these fractions and also from the unfractionated supernatant were tested for the incorporation of [^{14}C]leucine and the data obtained were compared with the nucleotide composition of these RNA preparations. The treatment at pH 5.3 effectively precipitates only the inactive RNA, leaving the active RNA in the supernatant. At pH 5.0 a part of the active RNA was precipitated along with the inactive RNA. Comparison of the incorporation data with those of the nucleotide composition analyses makes it clear that the ability to incorporate [^{14}C]leucine into different RNA's is directly proportional to their content of pseudouridylic acid, as may be seen in Table II, Expt. 2. The most active RNA (from the pH 5.0 or 5.3 supernatant) contained about 5.6 % of this nucleotide. Only a trace of this was found in the inactive RNA (from the pH 5.3 precipitate). The RNA with intermediate activity (from the pH 5.0 precipitate) contained 1.3 % pseudouridylic acid. Comparison of the active with the inactive RNA, showed that the former had higher contents of cytidylic and lower adenylic and uridylic acids

The higher cytidylic acid content in the active RNA may be attributed in part to the terminal -pCpCpA group[9], although the occurrence of this group in our materials has not yet been examined. As Table II shows, another point of interest in the nucleotide composition is the fact that the active RNA contains less uridylic acid and more pseudouridylic acid and that the sum of these two nucleotides is nearly constant throughout these RNA preparations. The data strongly suggest that the pseudouridylic acid is a unique constituent of the RNA active for the incorporation of amino acids (at least leucine) and that some part of the uridylic acid in the active molecule is replaced by pseudouridylic acid.

There is a good deal of evidence now which indicates that amino acid is linked to the RNA which is specific for each amino acid[9, 11, 30, 31]. It is therefore interesting to find out whether the relation of the nucleotide composition of RNA's to the leucine incorporation evidenced in this study also holds when other amino acids are used. Investigation on this question is now in progress.

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REFERENCES

[1] G. W. Crosbie, R. M. S. Smellie and J. N. Davidson, *Biochem. J.*, 54 (1953) 287.
[2] G. De Lamirande, C. Allard and A. Cantero, *J. Biol. Chem.*, 214 (1955) 519.
[3] D. Elson, L. W. Trent and E. Chargaff, *Biochim. Biophys. Acta*, 17 (1955) 362.
[4] S. Osawa, K. Takata and Y. Hotta, *Biochim. Biophys. Acta*, 28 (1958) 271.
[5] R. Jeener and D. Szafarz, *Arch. Biochem.*, 26 (1950) 54.
[6] R. M. S. Smellie, W. M. McIndoe, R. Logan and J. N. Davidson, *Biochem. J.*, 54 (1953) 280.
[7] C. P. Barnum and R. A. Husebey, *Arch. Biochem.*, 29 (1950) 7.
[8] M. B. Hoagland, M. L. Stephenson, J. F. S. Scott, L. I. Hecht and P. C. Zamecnik, *J. Biol. Chem.*, 231 (1958) 241.
[9] L. I. Hecht, M. L. Stephenson and P. C. Zamecnik, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 505.
[10] H. G. Zachau, G. Acs and F. Lipmann, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 885.
[11] J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann and M. Dieckmann, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 319.
[12] E. Otaka, Y. Hotta and S. Osawa, *Biochim. Biophys. Acta*, 35 (1959) 266.
[13] S. Osawa and E. Otaka, *Biochim. Biophys. Acta*, 36 (1959) 549.
[14] H. Halvorson and S. Spiegelman, *J. Bacteriol.*, 64 (1952) 207.
[15] F. Chao and H. Schachman, *Arch. Biochem. Biophys.*, 61 (1956) 220.
[16] I. Watanabe, *J. Gen. Physiol.*, 40 (1957) 521.
[17] A. Tissières and J. D. Watson, *Nature*, 182 (1958) 778.
[18] E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.*, 221 (1956) 45.
[19] F. F. Davis and F. W. Allen, *J. Biol. Chem.*, 227 (1957) 907.
[20] K. S. Kirby, *Biochem. J.*, 64 (1956) 405.
[21] D. B. Dunn, *Biochim. Biophys. Acta*, 34 (1959) 286.
[22] P. L. Bergquist and R. F. F. Matthews, *Biochim. Biophys. Acta*, 34 (1959) 567.
[23] F. F. Davis, A. F. Carlucci and I. F. Roubein, *J. Biol. Chem.*, 234 (1959) 1525.
[24] J. W. Littlefield and D. B. Dunn, *Biochem. J.*, 70 (1958) 642.
[25] M. Adler, B. Weissmann and A. B. Gutman, *J. Biol. Chem.*, 230 (1958) 717.
[26] C. T. Yu and F. W. Allen, *Biochim. Biophys. Acta*, 32 (1959) 393.
[27] W. Cohn, *Biochim. Biophys. Acta*, 32 (1959) 469.
[28] G. Brawerman and E. Chargaff, *Biochim. Biophys. Acta*, 31 (1959) 164.
[29] G. Brawerman and E. Chargaff, *Biochim. Biophys. Acta*, 31 (1959) 171.
[30] K. C. Smith, E. Cordes and R. S. Schweet, *Biochim. Biophys. Acta*, 33 (1959) 286.
[31] R. W. Holley, B. P. Doctor and S. H. Merrill, *Biochim. Biophys. Acta*, 35 (1959) 272.

STUDIES IN PARTIALLY RESOLVED BACTERIOPHAGE-HOST SYSTEMS

VI. THE INVOLVEMENT OF RIBONUCLEIC ACID IN VARIOUS ASPECTS OF THE REPRODUCTION OF BACTERIOPHAGE T2

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SUMMARY

A variety of inhibitors has been shown to have pronounced effects on the replication of T2 bacteriophage in protoplasts of *E. coli* B. Ribonuclease applied in 5 or 10 min pulses exerts a maximum effect (40 % reduction of burst size) when applied between 5 and 10 min after infection. *p*-chloromercury benzoate shows a sharp maximum of effectiveness and inhibits the burst completely when added 15 to 20 min after infection. Of a variety of purine and pyrimidine antagonists, 5-fluorouridine and 2'-deoxy-5-fluorouridine showed the most striking effects. The latter compound appears to have a specific inhibitory action on DNA synthesis and the inhibition (some 98 %) is essentially completely reversed by thymidine. The action of 5-fluorouridine appears to be considerably more complex. In part its effect is also directly on DNA synthesis and is reversible by thymidine. During the first 10 min of infection, however, this inhibitor, especially when tested in the presence of thymidine, appears to exert its primary effect on RNA synthesis with resulting effects on the synthesis of "early protein" and hence DNA. These effects can be overcome by uridine. A combination of thymidine and uridine completely abolishes the inhibition by 5-fluorouridine even at quite high concentrations. These conclusions have been reached on the basis of measurements of phage yield and also of incorporation of $^{35}SO_4$ and $^{32}PO_4$ into the appropriate macromolecules.

INTRODUCTION

Current studies of the inter-relationships of genetically specific DNA, RNA and protein involve many different systems. The one which we are considering—T2 in-

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; π, the protoplast-infecting agent derived from T2 (see previous papers); RNase, ribonuclease; FUR, 5-fluorouridine; dFUR, 2'-deoxy-5-fluorouridine; UR, uridine; TR, thymidine; UMP, uridine-5'-phosphate; dUMP, deoxyuridine-5-phosphate; Tris, tris-(hydroxymethyl) aminomethane; PCA, perchloric acid; TCA, trichloroacetic acid; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; BSA, bovine serum albumin; PCMB, *p*-chloromercury benzoate; BBSA, nutrient broth containing 2 % BSA.

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fected *Escherichia coli*—offers many advantages. It is clear that phage DNA is the initiating carrier of specificity (see review by HERSHEY[1]) and that proteins and, presumably, RNA are involved specifically and obligatorily. The proteins now known to be required in the replication of T-even phages include the following: (a) The "early protein" apparently essential for DNA synthesis[2-5]. (b) Certain enzymes the synthesis of which is induced by phage infection[6-10], some or all of which may be identical with "early protein". (c) The various head, tail, and internal proteins of the phage particle[11,12]. The RNA of *E. coli* seems to be typical of RNA as involved in protein synthesis in a wide variety of biological systems in that it occurs in two forms, soluble and particulate, with distinct functions. The soluble RNA appears to serve as the carrier of individual amino acids during their initial activation[13,14]. The fixed RNA seems to be an integral part of the site of protein synthesis[15] and to be concerned with the control of the arrangements of individual units in polypeptide sequences. This last RNA, then, is presumably the bearer of the primary information during the process now called "patternization"[16-18]. There is as yet no unequivocal answer to the question of whether in the phage—*E. coli* system well-known to be novel in a number of particulars, there occurs direct transfer of information from phage DNA to new protein. The weight of evidence, however, now seems to favor RNA intermediacy. We have found a polyribonucleotide to be directly concerned in an apparent first step of the infection of protoplasts of *E. coli* by π[19]. A very early step, possibly succeeding this one, appears to involve activation of polynucleotide phosphorylase[20]. An RNA fraction which is capable of rapid incorporation and turnover of $^{32}PO_4$ occurs as a direct and immediate consequence of infection of *E. coli* by bacteriophage T2 as shown by VOLKIN AND ASTRACHAN[21,22].

The present report is concerned with attempts to establish additional relationships between the synthesis of RNA and protein at the various stages of phage reproduction. A variety of experimental approaches has been employed for this purpose. We have investigated (a) the effect on phage reproduction of PCMB and of RNase applied in short pulses; (b) the effect on phage reproduction of a variety of inhibitors of nucleic acid synthesis—notably purine, pyrimidine, and nucleoside analogues and antagonists, (c) the effect on the rates of synthesis of DNA, RNA, and protein of some of these same inhibitors applied during the first few minutes after infection.

METHODS AND MATERIALS

Except for the isotope experiments to be described below, the techniques used were either standard phage procedures[23], or, when involving π, our own methods which have already been described[24-26]. The particular details for each experiment are listed in the legend of the appropriate table or figure. The isotope experiments were performed essentially as described by HERSHEY AND MELECHEN[4] with the following modification: *E. coli* B was grown through 6 transfers in HERSHEY's low phosphate, low sulfate medium (herein abbreviated as H medium); the sixth growth flask was then used as a source of cells for all isotope experiments. Bacteria in 250 ml of medium were allowed to grow to a concentration of $2.0 \cdot 10^8$ cells/ml (actual Petroff-Hausser count), centrifuged and washed twice with cold 0.1 *M* Tris buffer pH 8.1. They were then suspended in 25 ml (concentration: $2.0 \cdot 10^9$ cells/ml) of modified H medium freed of sulfate (for the ^{35}S experiments) or phosphate (for the ^{32}P experiments). 2.5 ml of each of these cell suspensions were mixed with an equal volume of a solution, consisting of the modified H medium plus the following (final concentration after mixing): in tube 1 – 300 µg FUR/ml; tube 2 – 300 µg FUR and 300 µg uridine/ml; tube 3 – 300 µg FUR and 300 µg thymidine/ml; tube 4 – 300 µg FUR, 300 µg uridine and 300 µg thymidine/ml; in tube 5 – 300 µg dFUR; in tube 6 – 300 µg dFUR and 300 µg thymidine; in tube 7 – 25 µg chloramphenicol/ml and in tube 8 – no inhibitor. After 5 min at 0° the reaction tubes were placed in a 37° bath and aeration was started. T2 bacteriophage was then added in a serial fashion to give an average multiplicity of 10 T2/cell. (Absorption was 99 % complete and over 90 % of all cells were shown to be infected in parallel experiments.) After precisely 1 min $Na_2{}^{35}SO_4$ was added to one set and $Na_2H^{32}PO_4$ to the other. The amount of phosphate added to each tube corresponded to 8 µg P and, in our counter, showed $3.55 \cdot 10^8$ counts/min on the day of the experiment. The amount of sulfate added was approx. 10 µg S and, on the day of the experiment, registered $1.07 \cdot 10^7$ counts/min. After addition of the radioisotopes phage growth at 37°, with constant aeration, was allowed to proceed for precisely 6.5 min. The various sets were then treated as follows:

In the ^{35}S experiment the tubes were chilled, a sample of each was removed for radio-assay of total ^{35}S present (0.05 ml of a 1:3000 dilution per planchet), the remainder was centrifuged in the cold (Servall). The pellets were washed twice by re-suspension and re-centrifugation in 0.1 *M* Na_2SO_4 to dilute out any $^{35}SO_4^{=}$ not incorporated into the cellular material. Each final pellet was then taken up in 2.5 ml of cold protoplast medium (final concentration $2.0 \cdot 10^9$ cells/ml). A 1-ml aliquot of this suspension was removed, an equal volume of 1.0 *M* PCA was added, the mixture was allowed to stand in the cold for a period of 2 h and then centrifuged. The precipitates were resuspended in 1.0 ml of cold 0.5 *M* PCA and re-centrifuged. This process was repeated once more. After a dilution of 1:2000, 0.05-ml aliquots of each sample were spread on planchets and counted to obtain the total sulfur incorporated into cell proteins. The cells in a second 1-ml aliquot were osmotically shocked by conversion to protoplasts followed by a rapid dilution into 0.01 *M* NaCl with strong agitation (cell lysis > 99 % complete by direct microscopic count or disappearance of zero-time infective centers). The lysate was then centrifuged at 4500 × *g* for 10 min to obtain a low speed residue (P_1) and a supernatant. The latter was centrifuged at 15,000 × *g* for 20 min to yield a high-speed pellet (P_2) and the final supernatant (S). P_1 and P_2 were suspended in 1.0 ml of cold 0.5 *M* PCA and treated as described above for whole cells. The final dilutions for radio assay were 1:400 and 1:40 respectively. The S fraction was precipitated with an equal volume of 1.0 *M* PCA and then treated as above; final dilution for counting 1:400.

In the ^{32}P experiments the tubes were chilled, 0.5 ml of unlabeled 0.150 *M* KH_2PO_4 was added to each, an 0.1-ml aliquot was removed for radioassay of total ^{32}P (final plating – 0.05 ml of a $1:4 \cdot 10^5$ dilution) and the remainder was centrifuged. The pellets were washed twice with 0.1 *M* Tris containing 0.01 *M* KH_2PO_4 and taken up in 2.5 ml each of protoplast medium. Sample treatment, cell lysis, centrifugal fractionation and PCA precipitation were then performed as described above. The nucleic acids thus obtained were fractionated by a modified Schmidt-Thannhauser procedure as follows: the precipitates were dissolved in 2.0 ml of 1 *N* NaOH and incubated for 18 h at 36°. The solutions were then neutralized with 0.20 ml of 10 *M*

HCl and made 0.5 *M* in PCA by addition of 2.2 ml of *M* PCA. The residues were taken up in 4.4 ml each of PCA and plated; these constitute the "DNA" fractions. The supernatants, also in a volume of 4.4 ml, constitute the "RNA" fractions.

The bovine serum albumin (BSA) was Armour 30 % sterile solution. The RNase used was Worthington Laboratories twice recrystallized product, the PCMB was purchased from Sigma Chemical Company and the various purines and pyrimidines were obtained from Schwarz Laboratories or from the California Corporation for Biochemical Research and were chromatographically pure. 8-azaguanine, 2-thiouracil and 5-bromouracil all were products of the California Corporation for Biochemical Research. 5-hydroxyuridine was given to us by courtesy of Professor VISSER of the University of Southern California; generous supplies of FUR, dFUR, were provided through the courtesy of Drs. AESHLIMANN, RUBIN AND SCOTT of Hoffmann-La Roche, Inc., and of DRB through that of Dr. FOLKERS of Merck-Sharp and Dohme. Phosphate ^{32}P and sulfur ^{35}S were obtained from Oak Ridge National Laboratories.

RESULTS AND DISCUSSION

Phage replication in protoplasts

Since our original report on the reproduction of T2 in protoplasts infected with π (see ref. 25) our procedures have undergone several modifications. A typical growth curve of the virus under the exact conditions used in the present experiments is, therefore, reproduced in Fig. 1a. The data show not only the free phage released spontaneously but also the total obtained upon osmotic shock of the infected protoplasts. Several features appear to be of interest. (a) The infected protoplasts behave like infected cells to some extent in that phage appear first within the protoplasts, and are released only after some delay. At 25 min the first internal phage appear. The differential between internal and released phage is at maximum at about 60 min. (b) Meanwhile phage are being released at an exponential rate between 25 and 120 min. The mechanism for this release is unknown to us but we believe that it may be a spontaneous destruction of the somewhat unstable protoplasts. (c) After some 150 min almost all of the protoplasts seem to have lysed (direct microscopic observation) and, indeed, there is no increase in phage numbers upon osmotic shock of the system. (d) The continued production of phage after this time is not immediately explicable. We have reason to believe that it is possible that replication may occur within a more highly resolved particulate system no longer containing intact protoplasts. It is also possible, however, that the effect is only apparent and due to unavoidable observational artifacts of the rather delicate protoplast system.

Phage replication in protoplasts, as in cells, is dependent on the maintenance of an adequate supply of oxygen and is sensitive to the ordinary inhibitors of respiration and phosphorylation (Table I).

Inhibition by RNase

In previous publications we have shown that protoplasts of *E. coli*, especially in the presence of BSA and in a nutrient medium, may be exposed to the action of RNase without any apparent loss in numbers of bodies or deleterious effects on their gross morphology[24]. These observations were confirmed in the present instance for periods up to 15 min, even at 37° and at RNase levels as high as 500 μg/ml with

10^9 protoplasts/ml. In the experiments shown in Fig. 1-B infected protoplasts at $1\cdot10^7$/ml were exposed to the enzyme at 50 μg/ml for pulses of only 5 min during different stages of phage replication. The enzyme was then rendered innocuous by a 500-fold dilution, phage reproduction was allowed to continue for a total of 120 min, and the extent of inhibition was determined. It will be seen that the period of

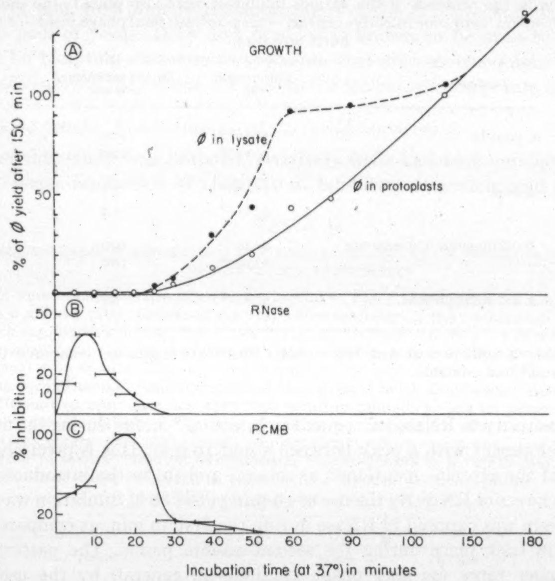

Fig. 1A. Growth of bacteriophage T2 in protoplasts of *E. coli* B, infected with π: Protoplasts at $2\cdot10^9$/ml were infected with an equal volume of a concentrated π preparation (nominal infective titer $2\cdot10^9$ infective units/ml). After 5 min at room temperature an aliquot of the mixture was diluted 1:1000 into a growth tube containing BBSA, held at 37°, and aerated vigorously. At the times indicated, samples were diluted 1:100 into BBSA ("phage in protoplasts") or distilled water ("phage in lysate") and plated. Actual plaque counts (calculated back to original infection tube, *i.e.* a dilution factor of $10\cdot10^2\cdot10^3$ for the zero time experiment) were: 0-time, $3.20\cdot10^8$/ml; 150 min (protoplasts) $3.40\cdot10^{10}$/ml; 150 min (lysate) $3.49\cdot10^{10}$/ml. Fig. 1B. RNase pulse experiment: Infected protoplasts (at 10^9/ml) were diluted 1:10 into BBSA and aerated vigorously at 37°. At the times indicated at the left hand margin of each set, samples from the concentrated growth tube were diluted 1:10 into BBSA containing 50 μg/ml of RNase. After 5 min exposure to the enzyme a dilution of 1:500 was made to stop enzyme action. (In separate experiments it was shown that RNase at this level (0.1 μg/ml) had no effect on overall phage production in protoplasts.) In this dilute growth tube, aeration was continued for a total of 90 min. An aliquot was then diluted and plated for phage. In a typical experiment, *e.g.* that represented by the third set, the protoplasts would have remained 10 min in the concentrated growth tube, 5 min (10–15 min from 0) in the RNase tube, and then an additional 75 min (15–90 min from 0) in the dilute growth tube. Data are expressed as percent inhibition of the phage yield in a control sample treated identically but devoid of RNase. Data for this control (calculated back to the original infected protoplasts) zero time infective centers—$3.00\cdot10^8$/ml; final phage yield—$2.40\cdot10^{10}$/ml; burst size, 80. Fig. 1C. PCMB pulse experiment: Similar to experiment 1B, except that the inhibitor used was PCMB at the level of $2\cdot10^{-4}$ *M*. The time of exposure was 5 min, as before; the dilution to stop inhibitor action was 1:1000; total incubation time —60 min. Data for the no-inhibitor control (calculated back as in Expt. 1B): zero time infective centers —$1.06\cdot10^8$/ml; final phage yield $2.30\cdot10^9$/ml; burst size, 22.

TABLE I

EFFECT OF METABOLIC INHIBITORS ON DEVELOPMENT OF T2 IN π-INFECTED PROTOPLASTS

Protoplasts were infected as described in the legend to Fig. 1. After 5 min an aliquot was diluted $1:2.5 \cdot 10^4$ into a growth tube containing the respective inhibitor at the concentration shown, and incubated at 37° for 2 h. At the end of this period an aliquot was suitably diluted, plated for phage, and the burst size compared to a control devoid of inhibitor. Controls for zero time infective centers in the presence of the various inhibitors were also plated—no inhibition was found. Data for control: zero time infective centers—$2.15 \cdot 10^8$/ml; final phage yield—$2.69 \cdot 10^{10}$/ml; burst size, 125.

Inhibitor	*Concentration (M)*	*Per cent inhibition of burst size**
Cyanide	$5 \cdot 10^{-3}$	100
	$5 \cdot 10^{-4}$	100
Azide	$5 \cdot 10^{-3}$	78
	$5 \cdot 10^{-4}$	13
p-chloromercuribenzoate	$2 \cdot 10^{-3}$	100
	$2 \cdot 10^{-4}$	100
2,4-dinitrophenol	$5 \cdot 10^{-4}$	91

* Plaques after 2 h/plaques at zero time, under identical conditions; compared to the same ratio for an uninhibited control.

maximum sensitivity to RNase subsequent to infection[19] occurs during the first 10 min of phage development with a peak between 5 and 10 min. This is precisely the time of synthesis of the proteins mentioned as sets (a) and (b) in the introduction and of maximum turnover of RNA. By the use of 10-min pulses 80 % inhibition was observed when the system was exposed to RNase during the first 10 min, as compared to 20 % when exposure took place during the second 10-min period. The pattern may be shifted to longer times (as may phage synthesis in general) by the use of lower temperatures. At 22° the extent of inhibition after enzyme treatment for the first 10 min was only 5 %; this was increased to 39 % during the second 10 min. This selective action of RNase on some process specific for phage replication, and presumable related to the synthesis of some specific protein(s) confirms recent findings and hypotheses advanced by JEENER[27] who used an induced, lysogenic strain of *Bacillus megaterium*.

Inhibition by PCMB

A similar pulse experiment with PCMB, a known inhibitor of many respiratory enzymes as well as of infection by intact T2 (see ref. 19), is shown in Fig. 1C. Although its inhibitory action, at $2 \cdot 10^{-4}$ *M* concentration is exerted throughout the first 25 min or so of phage development, there is a sharp and pronounced maximum between 15 and 20 min after infection. Experiments with 10-min pulses of exposure to inhibitor covering the entire period of phage development have yielded qualitatively similar results with maximum inhibition between 10 and 20 min. The greatly decreased and ever decreasing inhibition in the subsequent period (when phage is assembled at a maximal rate) is especially surprising. The result suggests tentative exclusion of phage protein itself as a possible locus of action of the inhibitor. Excluded also are the energy yielding enzyme sequences of the host, since they too would be expected to be taxed to their full capacity during the period of active phage synthesis and assembly commencing at 25 min. The action of the inhibitor may therefore be localized first of all on some protein or proteins concerned with the initial phases of phage replication, and, even more profoundly, on one (or more) which makes its appearance at about 15 min, just prior to the appearance of the first progeny phage at a time when the pool of phage DNA and proteins is known to be already fully formed. It would be tempting therefore to speculate that this protein might be concerned with the assembly of the various components of the bacteriophage into an intact virus.

Inhibition by purine, pyridimidine and nucleoside antagonists

When this study was initiated, relatively little had been published about the effects on phage replication of a variety of inhibitors of nucleic acid biosynthesis[§].

TABLE II

EFFECT OF VARIOUS PURINE AND PYRIMIDINE ANALOGUES ON DEVELOPMENT OF T2 IN π-INFECTED PROTOPLASTS

Protoplasts were infected as described in the legend to Fig. 1. After 5 min an aliquot was diluted 1:500 into a growth tube containing the respective inhibitor at the concentration shown. After 30 min each sample was divided into three parts, one, labeled "30 min", was diluted 1:500 into BBSA; the second, labeled "60 min-diluted", was diluted 1:50 into BBSA; the third was retained in the original growth tube. Thus the first contained the inhibitors at 0.002 the original concentration, the second at 0.02 and the third at the original level. Incubation was then continued for an additional 30 min. Samples were then suitably diluted, plated for phage, and compared to controls treated similarly but devoid of inhibitor. Controls for effect of inhibitors on zero time infective centers were also plated—no significant effect was found. The table represents data collected in several different experiments. Plaque counts in a typical experiment for the controls: zero time infective centers —$2.88 \cdot 10^8$/ml; "30 min" —$2.10 \cdot 10^{10}$/ml; "60 min diluted" —$2.10 \cdot 10^{10}$/ml; "60 min undiluted" —$1.96 \cdot 10^{10}$/ml.

Inhibitor	*Concentration (mM)*	*% inhibition* after exposure for*		
		30 min	*60 min*	
			Diluted	*Undiluted*
8-azaguanine	1	0	43	53
	5	76	81	95
2-thiouracil	1	0	29	32
	5	72	75	90
5-OH uridine	1	0	13	32
	5	84	83	90
5-Br uracil	0.5	0	53	—
	1	90	99	99
dFUR	0.5	67	22	72
	1	—	—	98
FUR	0.5	76	56	90
	1	90	—	95
DRB	1	82	82	95

* Plaques at end of incubation period/plaques at zero time, in this particular experiment; compared to control treated identically but devoid of inhibitor.

[§] These investigations were undertaken before the publication of the brilliant study of COHEN *et al.*[37] which localized the main bacteriocidal locus of 5-fluorouracil, FUR and dFUR at the level of the thymidylate synthetase, with dFUR-5′-phosphate as the actual inhibitory species.

We therefore decided to screen a variety of such agents using the π-protoplast system, which has the particular advantages of accessibility of the metabolic apparatus to charged molecules, *e.g.* ATP. The results of these experiments are summarized in Table II. Among the agents tested, the two fluorinated nucleosides, FUR and dFUR appeared to exert their action at the lowest concentration and were therefore selected for further study. It also seemed of interest that of all the inhibitors studied only the effects exerted by the fluoropyrimidine nucleosides could be reversed, at least in part, by further incubation in nutrient media. (Compare the per cent inhibition after "30 min" with that after "60 min diluted".) There was, therefore, the possibility that in these instances we were dealing with true inhibition of nucleic acid biosynthesis at the nucleoside or nucleotide levels, rather than with the formation of a "unnatural" nucleic acid which contained the analogue in its actual base structure. A survey of the various agents tested here with respect to their probable mechanism of action has recently been published by MATTHEWS[28]. Of these 8-azaguanine, 5-thiouracil, and 5-bromouracil all have indeed been shown to give rise to "unnatural" or modified nucleic acids, while 5-bromouracil, especially, has been reported to be incorporated into *E. coli*[29–31] and its bacteriophages[29] with powerful and specific mutagenic action[32, 33]. The other compounds tested were 5-hydroxyuridine, reported by ROBERTS AND VISSER[34] and by SPIEGELMAN[35] to block RNA synthesis in competition with uridine. DRB, which has similar action as an antagonist, has been reported by TAMM to be a competitive inhibitor of the incorporation of adenosine into RNA[36].

Inhibition of phage synthesis by FUR and dFUR

In order to define the locus or loci of action of the fluorinated pyrimidine nucleosides we have examined their effect on the reproduction of T2 in cells and protoplasts and in various media. These include a nutrient broth—BSA medium (BBSA) standard for our experiment with protoplasts, a synthetic medium (D) and a minimal synthetic medium (H), developed by HERSHEY specifically for incorporation studies with inorganic phosphate and sulfate. We have also attempted to localize their mode of action by means of a study of the reversibility of the inhibition by a variety of different compounds such as nucleosides, etc. Some typical experiments are summarized in Table III. Several different conclusions have emerged from these studies: (a) With the inhibitor added in millimolar concentration, inhibition of phage synthesis can be observed in all media and in both cells and protoplasts. (b) As might be expected the inhibition is more pronounced in a synthetic than in a broth medium; experiments with the minimal synthetic medium show the most dramatic effects. (c) At equal concentration, the riboside and deoxyriboside give rise to approximately equal inhibition, but the pattern for the reversal of this inhibition is grossly different for the two agents. (d) Among the compounds tested, only pyrimidine nucleosides appear to be capable of overcoming the inhibition exerted by the fluorinated derivatives. (e) Inhibition by FU is overcome in an apparently competitive manner by uridine and in a non-competitive manner by thymidine; addition of both gives a synergistic effect. (f) Inhibition by dFU is reversed completely by the addition of thymidine; uridine has little or no effect if added either alone or in conjunction with thymidine. Since thymidine reverses FUR inhibition only partially but dFUR inhibition completely, it would seem that FUR affects two different sites and dFUR only one of these. This latter site is probably concerned with DNA synthesis.

TABLE III

EFFECTS OF FUR AND dFUR ON DEVELOPMENT OF T2 IN CELLS INFECTED WITH T2 AND ON PROTOPLASTS INFECTED WITH π

The experiments with protoplasts were performed in a manner similar to that described in the legend to Table II (but with the inhibitor present throughout the growth period). Incubation in the presence of the inhibitor plus reversing agent shown was for a total of 90 min; there were no significant effects of the addition of these agents on the burst size of the control, average ~ 60, determined separately for each experiment. In the case of cells, growth was in the medium indicated to a concentration of $2.0 \cdot 10^8$ bacteria/ml. After washing with 0.1 *M* Tris, the cells were resuspended in the growth medium to $1.0 \cdot 10^9$ cells/ml and infected with T2 at an average multiplicity of 8 phages/bacteria. Adsorption was measured in each experiment by plating the supernatants after centrifuging and shown to be 99% complete. Infection was allowed to proceed for 5 min. The cells were then chilled, centrifuged, washed twice with 0.1 *M* Tris and resuspended in 25 ml of cold medium. Plating for infective centers indicated that all the cells (10^9/ml) had become infected. Aliquots were then diluted 1:200 into growth tubes containing the respective inhibitors plus reversing agents. Incubation was for 90 min at 37° and the values shown are the percentage of burst size when compared to controls devoid of inhibitor. Burst size of control in D, 130; in H, 75. In the experiment in H medium which constituted the phage growth control for the isotope experiments, the following procedure was followed: after centrifuging and washing the cells were resuspended in cold H medium to a concentration of $2 \cdot 10^9$ cells/ml. 1.0-ml samples were then mixed with equal volumes of the various agents to give the final concentrations shown. Aliquots were plated for zero time infective centers, and the tubes placed in a 37° bath with constant aeration. After 90 min samples were removed, diluted $1:10^6$ and $1:10^7$ and plated for phage. Calculation of % inhibition was done as before on the basis of burst size: burst size of control, 50.

System				*Cells*		*Protoplasts*
Medium				*D*	*H*	*B-BSA*
Inhibitor		*Reversing agent*		*% of untreated control**		
Compound	*Concentration (M)*	*Compound*	*Concentration (M)*			
FUR	$1.0 \cdot 10^{-3}$	None		10		
		Uridine (UR)	$1.0 \cdot 10^{-3}$	24		
		Thymidine (TR)	$1.0 \cdot 10^{-3}$	57		
		TR + UR	$1.0 \cdot 10^{-3}$	100		
	$1.14 \cdot 10^{-3}$	None			0.27	
		UR	$1.23 \cdot 10^{-3}$		1.2	
		TR	$1.24 \cdot 10^{-3}$		0.30	
		TR + UR	$1.23 \cdot 10^{-3}$ ea.		95	
	$7.68 \cdot 10^{-4}$	None				4.7
		UR	$1.0 \cdot 10^{-3}$			11
			$3.0 \cdot 10^{-3}$			15
			$5.0 \cdot 10^{-3}$			24
			$1.0 \cdot 10^{-2}$			36
		TR	$1.0 \cdot 10^{-3}$			15
			$3.0 \cdot 10^{-3}$			17
			$5.0 \cdot 10^{-3}$			20
			$1.0 \cdot 10^{-2}$			15
		Adenosine	$3.0 \cdot 10^{-3}$			6.7
		Guanosine	$3.0 \cdot 10^{-3}$			5.8
		Cytidine	$3.0 \cdot 10^{-3}$			5.2
	$1.0 \cdot 10^{-3}$	None				10
		UR	$5.0 \cdot 10^{-3}$			45
		TR	$5.0 \cdot 10^{-3}$			55
		Uracil	$5.0 \cdot 10^{-3}$			20
		Thymine	$5.0 \cdot 10^{-3}$			10

TABLE III (continued)

System				Cells		Protoplasts
Medium				D	H	B-BSA
Inhibitor		Reversing agent		% of untreated control*		
Compound	Concentration (M)	Compound	Concentration (M)			
dFUR	$1.0 \cdot 10^{-3}$	None		2.1		
		UR	$1.0 \cdot 10^{-3}$	3.3		
		TR	$1.0 \cdot 10^{-3}$	93		
		TR + UR	$1.0 \cdot 10^{-3}$	98		
	$1.14 \cdot 10^{-3}$	None			0.22	
		TR			59	
	$1.0 \cdot 10^{-3}$	None				10
		UR	$5.0 \cdot 10^{-3}$			9.6
		TR	$5.0 \cdot 10^{-3}$			75
		Uracil	$5.0 \cdot 10^{-3}$			11.2
		Thymine	$5.0 \cdot 10^{-3}$			22

* Plaques at end of incubation/plaques at zero time, under identical conditions; compared to the same ratio for a control devoid of inhibitor.

Our opinion of the function of the remaining site of FU inhibition is indicated by the following discussion based on the reactions of Fig. 2 which represents the currently accepted pathways of incorporation of uridine into RNA and DNA: FUR, or its nucleotide (FUR-5′-P) cannot cause inhibition solely at step (e) since in this case thymidine should reverse the action completely and competitively. Also there would then be no difference in action between FUR and dFUR. Conversely if action were exclusively at step (d), the action of thymidine would then be to reverse the inhibition non-competitively and completely. This indeed was the effect observed with dFUR. This then pinpoints the site of action of dFUR and is consistent with the hypothesis of COHEN[37] for the mode of action of fluorinated pyrimidines. Furthermore uridine should reverse inhibition of step (d) by FUR completely and competitively which is observed, and this reversal should be strictly additive to that exhibited by thymidine, which is not. Step (d) is the one postulated above, however, as being affected by both FUR and dFUR. If the second site for FUR action were steps (a) (as suggested by SKOLD[38]) and/or (c), thymidine should again reverse the inhibition non-competitively, and an additive effect of thymidine and uridine would be expected.

$$\begin{tikzcd}
\mathrm{UR} \arrow[r, "(a)"] & \mathrm{UMP} \arrow[r, "(b)"] \arrow[d, "(c)"] & \mathrm{RNA} \\
\mathrm{dUR} \arrow[r] & \mathrm{dUMP} \arrow[d, "(d)"] & \\
\mathrm{TR} \arrow[r] & \mathrm{TMP} \arrow[r, "(e)"] & \mathrm{DNA}
\end{tikzcd}$$

Fig. 2

This hypothesis also is not capable therefore of explaining the powerful synergistic effect observed in H medium. This last effect implicates some reaction between UMP and RNA (*i.e.* sequence (b)) as the additional locus of FUR inhibition[38, 39], and of necessity suggests that the product (RNA) is essential for some step in phage replication. In order to establish this phenomenon with more precision, the following two sets of experiments have been performed.

Effects of delayed addition of dFUR and FUR

As can be seen from Table IV, if the addition of the fluoropyrimidine nucleosides was delayed for 5–10 min several interesting effects were observed: the extent of inhibition of phage replication was the same, but the reversal of FUR inhibition by thymidine was enhanced and the effect now appeared quantitatively similar to that obtained with dFUR. Thus after the first few minutes, inhibition of phage replication by FUR becomes more similar to that by dFUR and appears to be due largely to interference with DNA synthesis. As a corollary the main effect of FUR on RNA-metabolism, then, must occur during the first few minutes after phage infection, during the period of synthesis of the "early protein".

TABLE IV

EFFECT OF DELAYED ADDITION OF FUR AND dFUR

Experiment similar to that described in Table II (except for the dilutions after 30 min) for the samples marked "0"; in another set of samples those marked "+10" or "+5", the infected protoplasts were diluted 1:50 into BBSA and were incubated for 10 or 5 min at 37°. At the end of this period they were then diluted 1:10 into BBSA containing the agents shown and incubation was continued for 90 min. In Expt. I, the burst size was 50; in Expt. II, 65; in Expt. III, 62.

Expt.	*Inhibitor*	*Time added (min)*	% *Inhibition** Nucleosides added (at $5.0 \cdot 10^{-3}$ M)		
			None	*Uridine*	*Thymidine*
I	FU	0	95	75	72
		+10	95	—	51
	dFU	0	95	93	52
		+10	95	—	53
II	FU	0	94	—	66
		+ 5	90	—	31
III	FU	0	96	—	74
		+ 5	93	—	66
	dFU	0	98	—	56
		+ 5	90	—	56

* Plaques at end of incubation/plaques at zero time under identical conditions; compared to the same ratio for a control devoid of inhibitor.

Incorporation experiments

The above hypothesis predicts a strong effect of FUR on incorporation into infected cells, during the first few minutes, of [^{35}S]sulfate into protein and [^{32}P]phosphate into nucleic acids. Experiments to test the hypothesis are summarized in Table V. The experimental procedure used was very similar to that pioneered by

TABLE V

INCORPORATION OF $^{35}SO_4$ AND $H^{32}PO_4$ INTO CELLULAR CONSTITUENTS IN THE PRESENCE OF FUR AND dFUR

Experiment described in the text. Amount of label fixed was calculated on a basis of 10^9 cells/ml.

Label added		*[^{35}S]sulfate*		*[^{32}P]phosphate*			
				Fraction of label fixed in		*% of control*	
Inhibitor	*Competitor*	*Fraction of label rendered PCA insoluble ($\times 10^{-3}$)*	*% of uninhibited control*	*DNA ($\times 10^5$)*	*RNA ($\times 10^4$)*	*DNA*	*RNA*
None	None	190	(100)	159	151	(100)	(100)
FUR	None	123	65	46	63	29	40
FUR	UR	183	96	57	109	36	72
FUR	TR	126	66	70	76	44	50
FUR	UR + TR	187	99	93	112	58	74
dFUR	None	142	75	62	110	39	73
dFUR	TR	127	66	79	79	50	53
Chloramphenicol	None	56	29	92	171	58	113

HERSHEY *et al.* Cells were multiply infected by incubation with 10 T2/cell for 1 min at 37° in a medium containing both the inhibitor (plus ordinary pyrimidine nucleoside where indicated) and the labeled substrate. Phage development was allowed to take place for an additional 6.5 min, at this temperature. Each sample was then chilled and analyzed. The effect of chloramphenicol at a level of 25 μg/ml was also studied in order to have available a comparison with an inhibitor of reasonably certain mode of action. It will be seen that FUR interferes, albeit to a varying extent, with all three of the incorporation proceses under investigation. Incorporation here is presumably to be equated with net synthesis of the respective components. The inhibition of RNA synthesis by FUR is, as demanded by the hypothesis advanced earlier, more effectively reversed by uridine than by thymidine. A similar inhibition and reversal pattern is also observed in the case of protein synthesis, presumably as a consequence of primary action on RNA synthesis. In agreement with the results of KIHO AND WATANABE[40, 41] and those of ASTRACHAN AND VOLKIN[22], published while this manuscript was in preparation, there is no inhibition, but rather a slight stimulation of the entry of phosphate into RNA in the presence of chloramphenicol. The small amount of DNA synthesis occurring during these early stages of virus replication (amounting to approximately only 0.1 the net synthesis of RNA) shows quite a different pattern. This pattern is, however, qualitatively in agreement with the results observed in the above experiments in which phage assay was used as a measure of inhibition. The effects include: partial reversal by thymidine of both dFUR and FUR inhibition; partial reversal by uridine of FUR inhibition; more complete and synergistic reversal by addition of both thymidine and uridine.

Structural localization of effects

In a previous paper of this series we have advanced a hypothesis of bacteriophage synthesis which assigns a paramount role to certain structural and functional elements of the bacterial cell, most notably the cell (or protoplast) membrane and the RNA associated therewith[19]. This hypothesis has since received added support by the findings of NISMAN[42] that the protoplast membrane of *E. coli*, as of other organisms, or possibly a class of ribosomes attached thereto[15] is implicated as the site of active protein synthesis. It therefore appeared to be of interest to see whether there were any readily discernible differences in the extent of isotope incorporation, subsequent to phage infection, into various fractions of a lysate (prepared as in our earlier papers by conversion of the cells to protoplasts by means of lysozyme–Versene and osmotic shock of the protoplasts*). Three fractions were examined: (a) P_1, a low speed residue, obtained after centrifuging for 10 min at 4500 × *g*. This fraction presumably contains any particulate cell-wall materials remaining, any intact cells and protoplasts, and intact protoplast ghosts and membranes. (b) P_2, a high speed residue obtained after centrifuging the remaining supernatant at 15,000 × *g* for 20 min. This fraction contains fragments of protoplast membranes and of wall material. (c) S_2, the supernatant remaining after (b). This contains all small particulate material and the soluble portion of the cytoplasm, including components falling into these classes liberated from the cells by action of lysozyme plus Versene.

In discussing the results obtained, which are summarized in Table VI, we shall deal separately with those bearing on protein and those bearing on nucleic acid synthesis. The more extensive data on protein synthesis (^{35}S incorporation) may be

TABLE VI

DISTRIBUTION OF ^{35}S AND ^{32}P IN VARIOUS FRACTIONS OF A PROTOPLAST LYSATE

Experiment described in the text. Amount of label fixed was calculated on a basis of 10^9 cells/ml.

Label added	*Incorporation into*	*Inhibitor*	*Competitor*	*Fraction of label fixed*			*% of uninhibited control*		
				P_1 ($\times 10^3$)	P_2 ($\times 10^4$)	S_2 ($\times 10^3$)	P_1	P_2	S_2
[^{35}S]sulfate	Protein	None	None	19.0	16.0	34.0	(100)	(100)	(100)
		FUR	None	12.3	4.67	29.6	65	29.4	87.0
		FUR	UR	23.0	18.1	41.8	120	111	123
		FUR	TR	11.5	5.20	31.6	60	32.5	93.0
		FUR	UR + TR	15.8	14.7	34.0	83	93	100
		dFUR	None	14.5	6.72	36.0	76	42.0	106
		dFUR	TR	10.5	8.27	22.0	55	52.0	64.7
		Chloramphenicol	None	10.0	0.02	26.6	53	0.12	78.2
[^{32}P]phosphate	RNA	None	None	4.30	3.87	2.43	(100)	(100)	(100)
		FUR	None	2.24	1.90	1.80	52	49.0	74.0
		dFUR	None	2.20	1.80	1.80	51	46.3	74.0
		Chloramphenicol	None	6.25	2.15	16.5	145	55.7	677
	DNA	None	None	0.25	0.29	0.11	(100)	(100)	(100)
		FUR	None	0.27	0.24	0.040	108	83	36
		dFUR	None	0.29	0.23	0.066	116	80	60
		Chloramphenicol	None	0.32	0.17	0.077	128	58	70

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* Under the conditions of the experiment, conversion to protoplasts could be shown to be 99.9% complete, while shocking into 0.01 *M* NaCl led to the disruption of > 99% of the protoplasts, as indicated by direct microscopic count, disappearance of zero-time infective centers, and liberation of material absorbing ultraviolet light at 260 mμ.

summarized as follows: Qualitatively the picture is similar to that obtained for gross incorporation into phage-infected cells—both FUR and dFUR inhibit, but the inhibition by the former agent is more complete and may be reversed by uridine but not by thymidine. Reversal by uridine alone, however, is now more effective than that exerted by a combination of this agent with thymidine. Quantitatively there is a striking gradation of all of these effects in the order $P_2 > P_1 \gg S_2$. Although no precise determinations of protein or RNA content have been made in these particular experiments, our experience has shown that the P_2 fraction never contains more than 10% of the total protein or RNA of the cells. Thus the incorporation, inhibition, and reversal picture with respect to total incorporation is a reflection of a similar, but possibly even more pronounced pattern of specific activities. It is also of interest that the experiment with added chloramphenicol shows a similar distribution; again the effect is more pronounced in the particulate fraction—here the incorporation into the P_2 protein appears by far the most sensitive. These observations are of interest in at least two contexts: (a) The differential sensitivity to chloramphenicol at 25 μg/ml appears to give strong support to the contention of ARONSON AND SPIEGELMAN[43] that this level of the antibiotic is insufficient to eliminate all protein synthesis. (b) The differential inhibition pattern appears to confirm the hypotheses advanced among others by BUTLER *et al.*[44], by BROWN AND BROWN[45], and by NISMAN[42] that relatively large particulate elements, probably identical with the protoplast membrane system, are the site of active protein synthesis in bacteria.

In the case of RNA synthesis we find an analogous pattern. The inhibitory action by the fluorinated pyrimidine nucleosides is exerted primarily on the RNA of the particulate fractions. This is in agreement with some preliminary data cited by VOLKIN AND ASTRACHAN[46] which indicated that such a fraction was the site of the RNA turnover during the early phases of phage synthesis. The present experiments also confirm their more recent finding that there is no inhibition but a stimulation of net incorporation of phosphate into RNA under the influence of chloramphenicol[47] (see also ref. 40, 41). Our data, however, suggest a definite functional heterogeneity of the various RNA fractions studied: The presence of the antibiotic led to a depression of RNA synthesis and/or turnover in the P_2 fraction. This effect is parallel to the complete inhibition of protein synthesis, shown above. Since it appears to be fairly well established that chloramphenicol does not interfere with RNA metabolism *per se*[48–51], this observation must then imply that the synthesis of some of the polyribonucleotide appearing during the period immediately after phage infection is dependent on protein synthesis in an obligatory manner. It is of interest that an amino acid dependent ATP-^{14}C incorporation induced by phage DNA has recently been discovered by G. BROWN*. The very pronounced stimulation of phosphate incorporation into the P_2 and especially the S_2 fraction is of interest in view of the hypothesis advanced by a number of workers[48–51] that the primary function of chloramphenicol is the "uncoupling" of protein and RNA synthesis with a block exerted in such a fashion as to lead to the accumulation of an unstable nucleic acid rather than a stable ribonucleoprotein. This unstable nucleic acid may then well be discharged into and accumulate in the soluble portion of the cytoplasm*. In agreement also with our own data on gross incorporation into the RNA of cells there is a pronounced stimulation of net incorporation under the influence of chloramphenicol.

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* Private communication: *cf.* also G. BROWN in *Structure and Function of Genetic Elements*, Vol. 12. Brookhaven National Lab. Symposia in Biology, 12, 1959, p. 47.

Relatively little can be said about the distribution data for DNA in view of the low total levels of activity observed. Yet the occurrence of a dFUR and FUR-insensitive DNA synthesis in some of the fractions and their differential sensitivity to these agents may be of more than passing interest.

CONCLUDING REMARKS

The present observations permit one to make some comments concerning two points: the possible mode of inhibition of T2 reproduction in *E. coli* by fluorinated pyrimidines, and the possible role of RNA and protein synthesis in phage replication. In agreement with the hypothesis of COHEN *et al.*[37] dFUR probably blocks DNA synthesis selectively and hence prevents phage reproduction. FUR, however, appears to produce at least two distinct effects[39]. One is analagous to the effect of dFUR and blocks phage reproduction by interference with DNA synthesis. The other is concerned with RNA metabolism and is competitively overcome by uridine.

There would seem to be two obvious ways in which this last effect could occur; faulty, non-functional RNA may be synthesized with fluorouracil in place of uracil or else the entry of UMP, and hence the synthesis of RNA itself, may be blocked. Incorporation of fluorouracil into RNA has been reported to occur in certain tumors[39] and tobacco mosaic virus (TMV)[52]. But in the latter case even substitution of 32 % of the uracil did not lead to impairment of the specific infectivity although the yield of virus was lowered. Thus the synthesis of RNA and/or protein was partially blocked, but the RNA produced was fully functional. The alternative explanation postulates a block of RNA synthesis by interference with the utilization of uridine with a concomitant or resultant decrease in protein synthesis. This interpretation is rendered more likely, not only by the results with TMV, and our own data, but also by observations on two additional systems. HOROWITZ *et al.*[53], working with a uracil-requiring mutant of *E. coli*, found that fluorouracil severely depressed the net synthesis of RNA and the incorporation of [^{14}C]adenine. Although some protein synthesis appeared to occur, this protein was probably non-functional as indicated by a complete absence of formation of inducible β-galactosidase. Fluorouracil also caused a block of RNA synthesis in a hepatoma[54]. In our system the FUR block on RNA synthesis probably manifests itself during two separate and distinct periods of phage reproduction: during the period immediately after infection, *i.e.* the time when the early proteins are synthesized, and addition of the agent leads to eventual interference with DNA synthesis as well, and at a period subsequent thereto when the phage proteins proper are laid down[1], and the agent presumably no longer affects the synthesis of DNA**. Thus FUR mimics the action of chloramphenicol and of certain amino acid analogues.

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[*] A very similar phenomenon has also been observed in our laboratories by Dr. A. HUNT. *Pseudomonas fluorescens* KB$_1$, while adapting to nicotinate also accumulates RNA in the S$_2$ fraction in the presence of chloramphenicol—in this system it could be shown that initial synthesis occurred in the protoplast membrane, followed by liberation of the freshly synthesized RNA into the S fraction.

[**] To eliminate all effects of FUR on DNA synthesis it will be advisable to carry out all future experiments in the presence of saturating levels of thymidine.

[***] See note added in proof.

Unlike these agents, however, its primary action is presumably on RNA synthesis or on a joint precursor of protein and RNA.

The present report provides some additional evidence for some of the postulates advanced in a previous paper of this series[19] and the scheme presented there. Various fractions isolated from infected lysates show markedly different rates of incorporation of ^{35}S and of ^{32}P. It appears even more likely now that the protoplast membrane system, which may include associated particulate elements, possesses complete functional autonomy for the synthesis of the proteins and nucleic acids required for phage reproduction. Our data here also indicate that the ribonucleoside polyphosphate pool postulated must be in very rapid metabolic equilibrium with some of its component entities, since free pyrimidine nucleosides and inorganic phosphate appear to be capable of entering the polyribonucleotide required for the synthesis of "early protein". Resort to the principle of Occam's razor suggests identity of this polyribonucleotide to the "RNA" of our scheme.

NOTE ADDED IN PROOF

More recent reports by Horonitz et al indicate, however, that the first alternative, *i.e.* incorporation, may be responsible for the observed effects. A very considerable portion of the total RNA-uracil (97 %) of *E. coli* B was replaceable by FU when the cells were grown for 21 h in the presence of the inhibitor. Concomitantly with this synthesis of RNA there was formation of protein; DNA synthesis was inhibited completely. As with the uracil-auxotroph the ability of lactose to induce β-D-galactosidase was almost entirely blocked in the presence of FU as was any further rise in the enzyme in cells which had been pre-induced; cells of a constitutive strain were also unable to form additional enzyme. Some of these effects were reversible by uracil[55,56]. Whether or not incorporation of the analogue into an "early RNA" is sufficiently rapid must, however, still remain an open question. In the case of aza-uracil, another pyrimidine analogue recently shown to interfere with phage replication at an early stage[57] the block is definitely believed to occur *prior* to the formation of RNA.

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REFERENCES

[1] A. D. Hershey, *Advances in Virus Research*, 4 (1957) 25.
[2] S. S. Cohen and C. B. Fowler, *J. Exptl. Med.*, 85 (1947) 771.
[3] K. Burton, *Biochem. J.*, 61 (1955) 473.
[4] A. D. Hershey and N. Melechen, *Virology*, 3 (1957) 207.
[5] J. Tomizawa and S. Sunakawa, *J. Gen. Physiol.*, 39 (1956) 553.
[6] J. G. Flaks and S. S. Cohen, *Biochim. Biophys. Acta*, 25 (1957) 667; *J. Biol. Chem.*, 234 (1959) 1501.
[7] A. Kornberg, S. B. Zimmerman, S. R. Kornberg and J. Josse, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 772.

[8] R. Sommerville, K. Ebisuzaki and G. R. Greenberg, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1240.
[9] J. F. Koerner and M. S. Smith, *Federation Proc.*, 18 (1959) 264.
[10] K. Keck, H. R. Mahler and D. Fraser, *Arch. Biochem. Biophys.*, 86 (1960) 85.
[11] A. D. Hershey, *Virology*, 4 (1957) 237.
[12] L. Levine, J. L. Barlow and H. Van Vunakis, *Virology*, 6 (1958) 702.
[13] J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergman and M. Dieckman, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 319.
[14] L. I. Hecht, M. L. Stevenson and P. C. Zamecnik, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 505.
[15] K. McQuillen, R. B. Roberts and R. J. Britten, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1437.
[16] B. A. Askonas, J. L. Simpkin and T. S. Work, *4th International Congress of Biochemistry, Vienna*, 1958.
[17] M. B. Hoagland, *Symposium VIII, 4th International Congress of Biochemistry, Vienna*, 1958.
[18] M. B. Hoagland, *Brookhaven Symposia in Biol.*, 12 (1959) 40.
[19] A. Shug, D. Fraser and H. R. Mahler, *Biochim. Biophys. Acta*, 36 (1959) 142.
[20] M. Liedke, H. R. Mahler and D. Fraser, *Abstracts 123rd Meeting Am. Chem. Soc., Boston*, 1959, 21 C.
[21] E. Volkin and L. Astrachan, *Virology*, 2 (1956) 149.
[22] L. Astrachan and E. Volkin, *Biochim. Biophys. Acta*, 29 (1958) 536.
[23] M. H. Adams, *Methods in Med. Research*, 2 (1950) 1.
[24] D. Fraser and H. R. Mahler, *Arch. Biochem. Biophys.*, 69 (1957) 166.
[25] D. Fraser, H. R. Mahler, A. Shug and C. A. Thomas, Jr., *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 939.
[26] H. R. Mahler and D. Fraser, *Virology*, 8 (1959) 401.
[27] R. Jeener, *Biochim. Biophys. Acta*, 32 (1959) 106.
[28] R. E. F. Matthews, *Pharm. Rev.*, 10 (1958) 359.
[29] D. B. Dunn and J. D. Smith, *Biochem. J.*, 67 (1957) 494.
[30] S. Zamenhof and G. Griboff, *Nature*, 174 (1954) 306.
[31] T. D. Price, P. B. Hudson, H. H. Hinds, R. A. Darmstadt and S. Zamenhof, *Nature*, 178 (1956) 684.
[32] R. M. Litman and A. B. Pardee, *Nature*, 178 (1956) 529.
[33] S. Benzer and E. Freese, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 112.
[34] M. Roberts and D. W Visser, *J. Biol. Chem.*, 194 (1952) 695.
[35] S. Spiegelman, in O. H. Gaebler, *Enzymes: Units of Biological Structure and Function*, Academic Press, New York, 1956, p. 67.
[36] I. Tamm, *Symposium Soc. Gen. Microbiol.*, 8 (1958) 178.
[37] S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1004.
[38] O. Sköld, *Biochim. Biophys. Acta*, 29 (1958) 651.
[39] C. Heidelberger, N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. J. Schnitzer, E. Pleven and J. Scheiner, *Nature*, 179 (1957) 663.
[40] I. Watanabe, Y. Kiho and K. Miura, *Nature*, 181 (1958) 1127.
[41] I. Watanabe and Y. Kiho, *Intern. Symp. on Enzyme Chem., Tokyo and Kyoto*, 1957, p. 410.
[42] B. Nisman, *Biochim. Biophys. Acta*, 32 (1959) 18.
[43] A. I. Aronson and S. Spiegelman, *Biochim. Biophys. Acta*, 29 (1958) 214.
[44] J. A. V. Butler, A. R. Crathorn and G. D. Hunter, *Biochem. J.*, 69 (1958) 544.
[45] G. L. Brown and A. V. Brown, *Symposia, Soc. Exptl. Biol.*, 12 (1958) 6.
[46] E. Volkin and L. Astrachan, in W. D. McElroy and B. Glass, *The Chemical Basis of Heredity*, The Johns Hopkins Press, Baltimore, 1957, p. 686.
[47] L. Astrachan and E. Volkin, *Biochim. Biophys. Acta*, 32 (1959) 449.
[48] F. E. Hahn, M. Schaechter, W. S. Zeglowski, H. E. Hopps and J. Ciak, *Biochim. Biophys. Acta*, 26 (1957) 469.
[49] F. C. Neidhardt and F. Gros, *Biochim. Biophys. Acta*, 25 (1957) 513.
[50] A. B. Pardee, K. Paigen and L. S. Prestidge, *Biochim. Biophys. Acta*, 23 (1957) 162.
[51] J. Horowitz, A. Lombard and E. Chargaff, *J. Biol. Chem.*, 233 (1958) 1517.
[52] M. P. Gordon and M. Staehelin, *J. Am. Chem. Soc.*, 80 (1958) 2340.
[53] J. Horowitz, J. I. Saukkonen and E. Chargaff, *Biochim. Biophys. Acta*, 29 (1958) 222.
[54] I. Melnick, A. Cantarow and K. E. Paschkis, *Arch. Biochem. Biophys.*, 74 (1958) 281.
[55] J. Horowitz and E. Chargaff, *Nature*, 184 (1959) 1213.
[56] J. Horowitz and E. Chargaff, *Federation Proc.*, 19 (1960) 350.
[57] A. B. Pardee and L. S. Prestidge, *Biochim. Biophys. Acta*, 37 (1960) 544.

FRACTIONATION OF THE INSOLUBLE MATERIAL OF *CHLORELLA* CELLS

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SUMMARY

A procedure is described for the fractionation of *Chlorella* cell material, labeled with ^{14}C, based on solvent extraction followed by serial digestion with several enzymes, and extraction of the breakdown products following each enzymic treatment. The cell material was divided into fractions containing the following substances or their breakdown products: water- and ethanol-soluble metabolic intermediates; lipids and pigments; ribonucleic acid; deoxyribonucleic acid; proteins and polysaccharides; some radioactivity remained in the residue after extraction.

There was some variation in the purity of the various fractions. Of the ethanol- and water-insoluble materials, the fractions prepared by the use of ribonuclease and proteolytic enzymes contained only those substances to be expected following treatment with these enzymes. The deoxyribonucleic acid fraction was contaminated with glucose and some unidentified substances. However, clear evidence was obtained for the incorporation of ^{14}C into thymine, a substance characteristic of deoxyribonucleic acid.

The polysaccharide fraction, after hydrolysis, contained some amino acids from residual protein, but these could easily be separated from the sugars by paper or column chromatography.

INTRODUCTION

Most of the past work to investigate the incorporation of $^{14}CO_2$ during photosynthesis has been restricted to short-term studies of the ethanol- and water-soluble materials[1]. However, there are many problems in which the patterns of incorporation of the labelled carbon into the ethanol- and water-insoluble materials would be relevant. Although many techniques are available for the quantitative analysis of the insoluble materials of plant cells (proteins[2,3], carbohydrates[2], fats[2], pigments[2], nucleic acids[2,4], etc.), there are no methods available, as far as we are aware, for the actual physical separation of the various constituents in order that they may be separately assayed for incorporated radioacitivity. Chemical analysis of the fractions may be

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performed in the presence of other fractions provided that a specific method exists for the determination of the particular fraction under investigation. This type of methodology is not applicable to radioactivity measurements. In the latter case the criterion by which a fraction is assayed is the same for all the fractions studied, viz. the radioactivity. It is therefore obvious that unless the fractions can actually be separated one from the other, no estimate can be made of the radioactivity incorporated into each one.

While purely chemical methods exist for the fractionation and separation of bacterial cell materials, such as the scheme devised by ROBERTS *et al.*[5] for the analysis of *Escherichia coli*, these methods tend not to be satisfactory for plant cells owing to the large amounts of polysaccharide present in the latter, which often interfere with the various acidic extraction procedures for such substances as nucleic acids. No suitable methods along these lines have yet been worked out for plant cells (see ref. 2, p. 17).

It was therefore decided to attempt to develop a fractionation scheme based on enzymic analysis, since a greater degree of specificity for each fraction could be expected than when using purely chemical extraction procedures. The present communication describes such a scheme which has proved to be reasonably suitable for *Chlorella* cells, though further investigation has shown that it cannot be applied without modification to all plant tissues. It consists essentially of the following steps: (a) the removal of the ethanol- and ether-soluble materials; this is followed by a successive treatment by the following enzymes: (b) ribonuclease (c) deoxyribonuclease, (d) trypsin and chymotrypsin alternately and (e) diastase.

METHODS

Growth and photosynthesis

Cells were grown in the continuous culture apparatus described by HOLM-HANSEN *et al.*[6]. The cells were harvested, washed with fresh nutrient solution, and 1–2 ml of wet-packed cells were resuspended in 60 ml of nutrient solution MYER'S medium[7] and placed in the "steady state apparatus" described by BASSHAM *et al.*[8] and WILSON AND CALVIN[9]. 1 ml of $NaH^{14}CO_3$ (0.026 M; 400 μC) was added to the suspension and the cells were allowed to carry on photosynthesis for 4 h at a light intensity of 3000 foot candles. At the end of this period the cells were centrifuged, resuspended in 2 ml of distilled water, and lyophilized.

A number of analytical procedures were investigated and the following has given the most satisfactory results. A summary of this procedure appears in Table I.

Soluble materials

A portion of the dried cells (40 mg) was homogenized in a Potter homogenizer with 6 ml of 80 % (v/v) ethanol, and the suspension subsequently heated in a bath of boiling water until the ethanol began to boil. The cell residue was removed by centrifugation, and was re-extracted with 2 ml of boiling 80 % ethanol. This extraction was repeated twice more. After determination of the radioactivity in each extract, they were pooled. The residue was next extracted with 5 ml of 20 % (v/v) ethanol for 2 min at 70°. This extraction was repeated twice more with 2-ml amounts of 20 % ethanol.

TABLE I

DISTRIBUTION OF ACTIVITY IN VARIOUS CHEMICAL FRACTIONS OF *Chlorella* CELLS AFTER PHOTOSYNTHESIS FOR 4 H WITH RADIO-BICARBONATE

After photosynthesis the cells were lyophilized and extracted with various solvents and enzymes in the sequence shown below. The values given are the percentages of the total radioactivity fixed by the *Chlorella* cells; this was $128.5 \cdot 10^6$ counts/min/40 mg of lyophilized cells.

Sample No.	*Extracted with*	*Temp.*	*Extraction period*	*Activity (% of total)*	*Total activity in whole fraction (% of total)*
Ethanol–water and ethanol–ether soluble fractions					
1	80 % ethanol	Boiling	—	27.48	31.21
2	80 % ethanol	Boiling	—	2.29	
3	80 % ethanol	Boiling	—	0.98	
4	80 % ethanol	Boiling	—	0.46	
5	20 % ethanol	70	2 min	4.70	5.81
6	20 % ethanol	70	2 min	0.68	
7	20 % ethanol	70	2 min	0.43	
8	Distilled water	70	5 min	0.56	0.72
9	Distilled water	70	5 min	0.16	
10	Ethanol–ether (3:1)	70	5 min	0.12	0.12
	A total ethanol soluble fraction				37.86
	A 1, ether-extract of A			15.64	—
	A 2, residue from A 1			22.22	—
Ethanol–water and ethanol–ether insoluble fractions					
11	Ribonuclease	37	6 h*	2.56	3.22
12	Distilled water rinse	—	—	0.29	
13	Ribonuclease	37	3 h	0.22	
14	Distilled water rinse	—	—	0.15	
15	Deoxyribonuclease	37	3 h**	0.47	0.79
16	$MgSO_4$ rinse	—	—	0.14	
17	Deoxyribonuclease	37	1 h	0.13	
18	$MgSO_4$ rinse	—	—	0.05	
19	Trypsin	37	39 h	27.17	49.53
20	Distilled water rinse	—	—	2.59	
21	Trypsin	37	24 h	2.67	
22	Distilled water rinse	—	—	0.62	
23	Chymotrypsin	37	24 h	8.19	
24	Distilled water rinse	—	—	1.41	
25	Chymotrypsin	37	24 h	3.32	
26	Distilled water rinse	—	—	0.92	
27	Trypsin	37	24 h	0.81	
28	Distilled water rinse	—	—	0.38	
29	Trypsin	37	24 h	0.31	
30	Distilled water rinse	—	—	0.18	
31	Chymotrypsin	37	60 h	0.69	
32	Distilled water rinse	—	—	0.27	
33	Diastase	37	20 h	3.57	6.04
34	Distilled water rinse	—	—	0.44	
35	Diastase	37	20 h	0.86	
36	Distilled water rinse	—	—	0.33	
37	Diastase	37	20 h	0.70	
38	Distilled water rinse	—	—	0.14	
39	Residue	—	—	8.34	8.34
Total recovered activity				105.98	

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* 3 h is the optimum period for the first ribonuclease extraction (see Table II).

** 1 h is the optimum period for the first deoxyribonuclease extraction (see Table II).

After the extractions with ethanol, the residue was extracted with 15 ml of distilled water for 5 min at 70°; this extraction of the residue was repeated once more with 6 ml of water. The next extraction of the residue was with 6 ml of ethanol–ether (3:1, v/v) for 5 min at 70°. The above extractions removed those substances from the cells which are usually considered as being in the ethanol- and water-soluble fractions: the soluble metabolites, pigments, and lipids.

The total ethanol–, water– and ethanol–ether extracts were combined and extracted 3 times with 5 ml of ether. The total ether extracts were combined and the activities of the ethanol–water-soluble and ether-soluble fractions were measured.

Nucleic acids

The residue from the previous extracts was treated for 6 h with 3 mg of ribonuclease dissolved in 3 ml of water at 37°. After 3 h an aliquot was removed for determination of radioactivity in the supernatant after centrifugation. After the 6 h extraction period, the suspension was centrifuged and the residue was washed with 3 ml of distilled water. The residue was treated with a further 3 mg of ribonuclease in 3 ml of distilled water for 3 h at 37°, and the residue was again washed with 3 ml of water.

Following treatment with ribonuclease, the residue was digested with 3 mg of deoxyribonuclease in 3 ml of 0.003 M $MgSO_4$ for 3 h at 37°. After 1 h of this digestion, an aliquot was removed for assay of the radioactivity in the supernatant after centrifugation. At the end of the 3-h digestion period, the suspension was centrifuged and the residue washed with 3 ml of 0.003 M $MgSO_4$. The digestion with deoxyribonuclease was repeated (2 mg of enzyme in 2 ml of $MgSO_4$ solution) for 1 h and the residue was again washed with 2 ml of $MgSO_4$ solution.

Protein

The residue from the deoxyribonuclease extractions was incubated at 37° for 39 h with 20 mg of trypsin dissolved in 2 ml of 0.01 M potassium phosphate buffer, pH 7.6. An aliquot was removed after 15 h for radioactivity determinations on the supernatant. The residue was washed with 2 ml of distilled water. The extraction with trypsin was repeated for a further 24 h at 37°, and the residue was again washed with 2 ml of water. The residue was next extracted with chymotrypsin: 20 mg of enzyme was dissolved in 2 ml of 0.01 M phosphate buffer, pH 7.6, and the extraction was allowed to proceed at 37° for 24 h. After washing with 2 ml of distilled water, the extraction with chymotrypsin (24 h) and the washing with water was repeated.

The residue was again extracted with 2 ml of trypsin solution in phosphate buffer for 24 h, followed by washing with 2 ml of water. The trypsin digestion was repeated once more (24 h), followed by another washing with 2 ml of water. A final extraction was made with 2 ml of chymotrypsin solution in phosphate buffer (60 h) and the residue was again washed with water.

Polysaccharides

After removal of the proteins, the residue was treated for 20 h at 37° with 90 mg of malt diastase dissolved in 3 ml of distilled water. 8 h after the beginning of the incubation period, an aliquot was assayed for radioactivity in the supernatant following centrifugation. The residue was washed with 2 ml of water. The extractions with diastase (for 20 h periods) were repeated twice more. A residue remained which was not further extracted.

Analysis of the fractions

The successive extractions for each fraction were pooled and an aliquot was analyzed by paper chromatography to check the nature of the substances removed from the cells by each enzymic treatment. All chromatograms were made on oxalic acid-washed Whatman No. 4 filter paper, and were developed in phenol–water in the first dimension and in *n*-butanol–propionic acid–water in the second dimension[10]. Radioactive substances were located by exposure of the chromatograms to Dupont X-ray film No. 507E. Substances were identified by their chromatographic positions, by cochromatography with known markers, and by other properties such as absorption of u.v. light and reaction with appropriate sprays.

All fractions extracted from the insoluble material gave unsatisfactory chromatograms* unless they were first subjected to acid hydrolysis. After several trial attempts, the following hydrolysis conditions were found to be the most satisfactory: (a) for the ribonucleic acid fractions: heated at 100° for 2 h with 1 *N* HCl; (b) for the deoxyribonucleic acid fraction: heated at 120° for 2 h with 6 *N* HCl; (c) for the protein fractions: 1 volume of solution heated for 16 h at 110° with 8 volumes of a mixture of equal parts of 10 *N* HCl and glacial acetic acid; (d) for the polysaccharide fractions: heated with *N* HCl for 3 h at 100°. After hydrolysis, each deoxyribonucleic acid and each protein sample was evaporated to dryness, redissolved in water, taken to dryness twice more, and finally dissolved in water and applied to the chromatogram.

Radioactivity determinations

Samples for assay of radioactivity were dried onto thin aluminium planchettes and counted with a thin-window Scott-type Geiger-Muller tube connected to a scaler. Substances counted directly from paper chromatograms were counted with a similar tube. The tubes were flushed continuously with a gas mixture consisting of 99.05 % (v/v) helium and 0.95 % (v/v) isobutane.

Enzymes

The enzyme preparations used were obtained from the following sources: ribonuclease (crystallized) and chymotrypsin (crystallized, salt-free) from Armour Laboratories, Chicago, Ill.; deoxyribonuclease (once crystallized) from Worthington Biochemical Corp., Freehold, N.J.; trypsin (twice crystallized; contains approximately 50 % $MgSO_4$) from Mann Research Laboratories, New York, N.Y.; malt diastase from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Quantitative results

In almost every case a single extraction with a solvent or an enzyme is not sufficient to achieve a quantitative extraction of the substances involved. However, even though repeated extractions remove lesser and lesser amounts with each successive treatment, it is extremely difficult to reach a stage where no material at all is removed with a further extraction[8]. For this reason it is necessary to select some limit beyond which a particular extraction would not be pursued. In practice each extraction was stopped when no more than about 0.5 % of the total radioactivity originally present in the sample of lyophilized cells was removed by a further treatment; for the nucleic acid fractions this limit was reduced to about 0.2 %.

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* The chromatograms were made unsatisfactory by immovable origins and streaking spots.

Table I shows the sequence of extractions and washings and gives the radioactivity found in each fraction expressed as a percentage of the total radioactivity present in the whole sample of lyophilized cells before extraction.

Extraction times

During the first extraction with ribonuclease, deoxyribonuclease, trypsin, and diastase, aliquots were removed from the reaction vessel at various times in order to determine the optimum extraction period for each of these enzymes (see METHODS section). Table II shows the activity extracted after various incubation periods with these enzymes. The results demonstrated that, while there was no added advantage in extending the extraction periods with ribonuclease and deoxyribonuclease for longer than 3 h and 1 h, respectively, extraction was not complete with trypsin after 15 h, and 24 h was adopted as the standard extraction period. Extraction with diastase was incomplete after 8 h, and 20 h was made the standard reaction period.

TABLE II

EFFECT OF THE LENGTH OF THE INCUBATION PERIOD ON THE EXTRACTION OF THE INSOLUBLE CELL MATERIAL BY VARIOUS ENZYMES

The values given are the percentages of the total radioactivity fixed by the *Chlorella* cells. See Table I for identity of the sample nos.

Sample No.	*Extracted with*	*Extraction period*	*Activity % of total*
11	Ribonuclease	3 h	2.43
11	Ribonuclease	6 h	2.56
15	Deoxyribonuclease	1 h	0.41
15	Deoxyribonuclease	3 h	0.47
19	Trypsin	15 h	19.61
19	Trypsin	39 h	27.17
33	Diastase	8 h	2.63
33	Diastase	20 h	3.57

Composition of the extracted fractions

Ethanol–water-soluble fraction: The nature of the compounds extracted by each procedure was investigated chromatographically. The ethanol–water-soluble fractions contained sugar phosphates, amino acids, organic acids, free sugars, pigments, and lipids. After extraction of this fraction with ether, the ether contained the pigments, lipids, and phospholipids. These fractions were not investigated further.

Ribonucleic acid fraction: Chromatography of this fraction after hydrolysis with *N* HCl showed the presence of ^{14}C, in (a) ribose (14.6 % of the total radioactivity in this fraction), (b) in adenine (10.9 %), (c) in guanine (16.4 %), (d) in uridine (7.7 %), (e) in uridylic acid (23.7 %), (f) in an unknown u.v.-absorbing spot (19.2 %), (g) a small amount in glucose (2.4 %) and (h) in two unknown spots and the origin (5.1 %) (Fig. 1). It was found that the proportion of labeled glucose appearing in this fraction

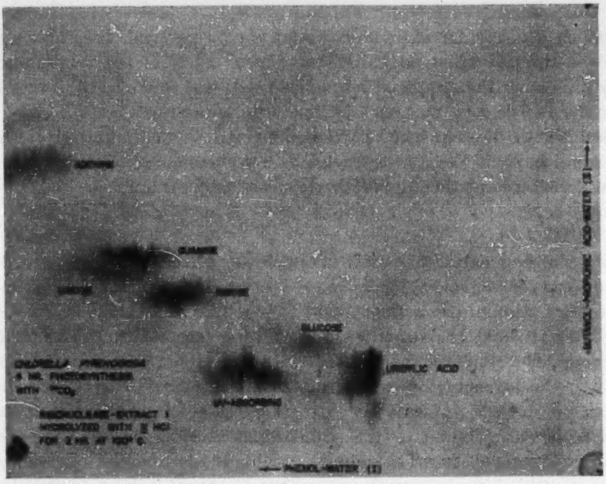

Fig. 1. Radioautogram of a chromatogram of the ribonuclease extract of *Chlorella pyrenoidosa* after hydrolysis with 1 *N* HCl for 2 h at 100° (4 h photosynthesis with $^{14}CO_2$).

increased considerably if the total extraction period with ribonuclease was prolonged for more than 6 h.

In another analytical procedure, cell residues, which had previously been extracted with ethanol–water and ethanol–ether, were extracted with 10 % (w/v) perchloric acid for 18 h at 4° (see ref. 4); this extract had the same qualitative and quantitative composition as extracts prepared with ribonuclease. Perchloric acid extracted 3.40 % of the total ^{14}C of the lyophilized cells compared with a total of 3.22 % in all the ribonuclease extracts and washings (Table I).

Deoxyribonucleic acid fraction: Very little ^{14}C was found in this fraction (Table I). While some of the constituents in this fraction, following hydrolysis, could be identified as nucleic acid components, this fraction was also contaminated with glucose and some unidentified compounds. Some of the radioactivity in this fraction coincided chromatographically with u.v.-absorbing substances which moved chromatographically in the positions of nucleosides, nucleotides and bases. As it was very difficult to decide whether the u.v.-absorbing materials originated from deoxyribonucleic acid, or residual ribonucleic acid, a specific search was made for the presence of thymine, the only purine or pyrimidine base characteristic of deoxyribonucleic acid as distinct from ribonucleic acid.

The isolation of deoxyribose is particularly difficult since chemical procedures to remove the phosphate from the deoxyribose nucleotides invariably destroy the sugar[12]; thymine was therefore used as the criterion of deoxyribonucleic acid extraction. Labeled thymine was indeed detected, indicating that this fraction did contain deoxyribonucleic acid although it was seriously contaminated with other substances. The following distribution of activity was found: thymine (4.7 %); adenine (2.8 %),

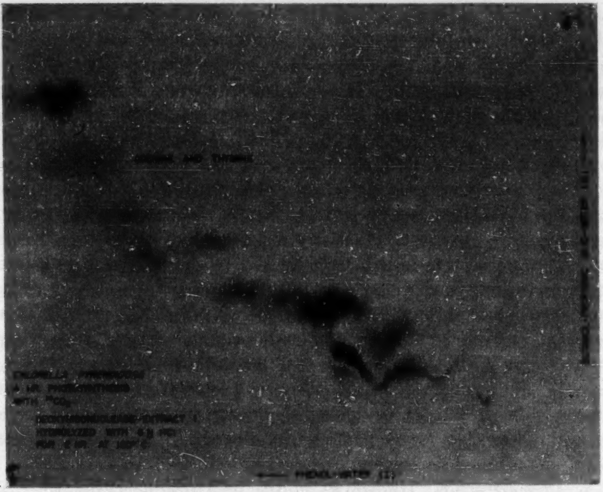

Fig. 2. Radioautogram of a chromatogram of the deoxyribonuclease extract of *Chlorella pyrenoidosa* after hydrolysis with 6 *N* HCl for 2 h at 120° (4 h photosynthesis with $^{14}CO_2$).

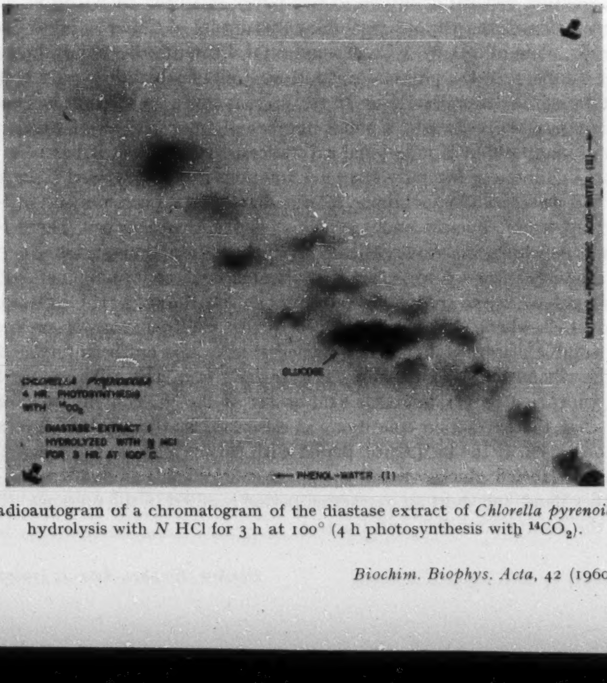

Fig. 3. Radioautogram of a chromatogram of the diastase extract of *Chlorella pyrenoidosa* after hydrolysis with *N* HCl for 3 h at 100° (4 h photosynthesis with $^{14}CO_2$).

other u.v.-absorbing substances (11.4 %); glucose plus levulinic acid (derived from glucose by acid treatment) (30.6 %); unidentified substances (51.5 %) (Fig. 2). Thymine and adenine are not well separated chromatographically with the solvents mentioned in the METHODS section. The mixed spot of thymine and adenine was therefore resolved by chromatography using water adjusted to pH 10 with ammonium hydroxide (see ref. 4, p. 252).

In order to detect free thymine vigorous hydrolysis conditions for deoxyribonucleic acid were necessary[13]. Under these conditions deoxyribose is destroyed[12].

Protein fraction: After hydrolysis this fraction consisted solely of amino acids: each radioactive area corresponded exactly with a ninhydrin-reacting area, and each ninhydrin-reacting area was radioactive. No differences were seen in extracts made with trypsin or with chymotrypsin, and the relative amounts of each of the amino acids remained constant through successive treatments with the proteolytic enzymes.

Polysaccharide fraction: This fraction contained, after hydrolysis, mainly glucose (37.4 % of the total ^{14}C in this fraction), together with some fructose (3.5 %), amino acids, (39.3 %), and some unidentified substances (19.8 %), one of which may have been a pentose (Fig. 3). The amino acids may be separated from the sugars either by treatment with Dowex 50, or by paper chromatography, and the relative amounts of ^{14}C in each determined separately.

Residue after extraction: Hydrolysis of the final residue either with *N* HCl or with a mixture of equal parts of 10 *N* HCl and glacial acetic acid demonstrated that most (65–85 %) of the residual ^{14}C was present in amino acids.

DISCUSSION

The fractionation method described in this paper had been shown to be applicable to the separation of the various insoluble constituents of *Chlorella* cells. There exist in algal cells, as in all others, a large number of different compounds, and different groups of compounds, the physical separation of all of which in a pure state would represent an enormous undertaking. In the present study an attempt has been made only to separate the cells into a small number of fractions, all of which must be mixtures of many different individual substances. Nevertheless, it has been possible to separate in a more or less pure state five fractions from ^{14}C labeled *Chlorella* cells: ethanol- and water-soluble metabolic intermediates; fats, pigments and other ether-soluble components; nucleic acids; proteins; and polysaccharides. There remained a small residue which was shown to contain ^{14}C mainly in amino acids.

The ethanol–water-soluble, ether-insoluble material, containing metabolic intermediates involved in the carbon reduction cycle, tricarboxylic acid cycle, etc., have been studied elsewhere[1] and have not been further considered in the present work; the ether-soluble materials (lipids and pigments) have also not been further fractionated[14]. The ribonucleic acid fraction, as extracted from the cell residues by ribonuclease, contained only ribonucleic acid as far as the ^{14}C content was concerned, provided that the extraction conditions as described in the METHODS section were properly observed. If the incubation period with ribonuclease was prolonged beyond a total of 6 h, labeled glucose began to appear in significant amounts in the extract. On the other hand, too short an extraction period resulted in the presence of labeled ribose in the deoxyribonucleic acid fraction.

The deoxyribonucleic acid fraction was the least satisfactory as regards purity. Some radioactivity was associated with thymine, indicating that the fraction indeed contained deoxyribonucleic acid. However, many contaminating substances were also present, and the hydrolysis conditions necessary for the isolation of thymine precluded the detection of deoxyribose. That the separation of RNA and DNA was not complete is demonstrated by the presence of a trace of thymine associated with the adenine from the RNA'se extract; the adenine-thymine area obtained from the DNA'se extract was largely thymine with some (1/3) adenine.

The alternate incubation with trypsin and chymotrypsin removed substances yielding only amino acids on hydrolysis. There was a distinct advantage in the use of two proteolytic enzymes for the extraction of protein (Table I). The main disadvantage of using enzymes to extract the protein was the very long incubation periods which had to be used. Even after a total incubation period with trypsin and chymotrypsin of 219 h, proteins were still removed in the subsequent diastase extractions, and amino acids were found in the hydrolysate of the final residue. It was considered easier to separate the amino acids from the sugars by paper or column chromatography after hydrolysis of the diastase extracts rather than to continue extraction with the proteolytic enzymes until no more ^{14}C appeared in amino acids.

The substances extracted by diastase, apart from some activity in amino acids as mentioned above, contained ^{14}C mainly in glucose. The presence of activity in fructose, and in an unidentified substance (probably a pentose from its chromatographic behavior) indicated the existence of polysaccharides other than glucose polymers in this organism.

The fractionation methods described in this communication have also been applied to the liverwort *Riella helicophylla*. The procedure was not satisfactory as early as the ribonucleic acid stage, since large amounts of labeled glucose appeared in this fraction after a relatively short incubation period with ribonuclease (3 h). This organism contains large amounts of starch, as can be seen by staining with iodine, and part of this starch may be rendered soluble by incubation with ribonuclease solution at 37°. Hence it may prove necessary to develop critical extraction procedures for each new plant species depending on the particular nature of the polysaccharide content.

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REFERENCES

[1] M. CALVIN, *J. Chem. Soc.*, (1956) 1895.
[2] K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1956.
[3] L. FOWDEN, *Biochem. J.*, 50 (1952) 355.
[4] E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Academic Press Inc., New York, 1955.
[5] R. B. ROBERTS, P. H. ABELSON, D. B. COWIE, E. T. BOLTON AND R. J. BRITTEN, *Carnegie Institution of Washington Publication*, Washington D.C., 1955, p. 607.

[6] O. Holm-Hansen, P. Hayes and P. Smith, *University of California Radiation Laboratory Chemistry Division Quarterly Reports*, UCRL-3595, 1956, p. 56.

[7] P. W. Krauss, in *Algal Culture from Laboratory to Pilot Plant*, Carnegie Institution of Washington, Washington D.C., 1953, publication 600.

[8] J. A. Bassham, K. Shibata, K. Steenberg, J. Bourdon and M. Calvin, *J. Am. Chem. Soc.*, 78 (1956) 4120.

[9] A. T. Wilson and M. Calvin, *J. Am. Chem. Soc.*, 77 (1955) 5948.

[10] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, *J. Am. Chem. Soc.*, 72 (1950) 1710.

[11] M. Ogur and G. Rosen, *Arch. Biochem.*, 25 (1949) 262.

[12] R. Feulgen, *Chemie und Physiologie der Nukleinstoffe*, Berlin, 1923.

[13] E. Chargaff, R. Libshitz, C. Green and M. E. Hodes, *J. Biol. Chem.*, 192 (1951) 223.

[14] U. Blass, *University of California Radiation Laboratory Chemistry Division Quarterly Report*, UCRL-3836, 1957, p. 46.

THE INFLUENCE OF STRUCTURE ON THE ORIENTATION OF THE VERATRUM ALKALOIDS AT THE AIR/WATER INTERFACE

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SUMMARY

The veratrum alkaloids—veracevine, veratridine, cevadine, and veratramine—have similar chemical structures yet are quite distinct in their pharmacological actions. Veracevine is inactive, veratridine and cevadine are "labilizers" or "unstabilizers", and veratramine is an antagonist for cevadine and veratridine. The properties of these compounds at the air/water interface also reveal marked differences. Surface tension measurements in water show surface activity decreases in the order veratramine $>$ veratridine $>$ cevadine $\gg$ veracevine. A Langmuir-type film balance was used to measure their F-A curves. Films of the alkaloids were unstable because of their slight solubility in the substrate, and a technique was devised which permits reproducible F-A measurements. Despite these precautions, veracevine did not form any films. The F-A curves for veratridine and cevadine show (a) an inflection which corresponds to their areas when oriented horizontally at the a/w interface, and (b) an area in which the molecules are tight-packed corresponding to molecular areas for the vertical orientation of the molecules. Veratramine shows no inflection in the F-A curve, but the tight-packed film occurs at an area in which this molecule is oriented almost horizontal to the a/w interface. Interpretation of these results is in terms of the distribution of hydrophobic and hydrophilic groups on the molecules; their implication for pharmacological action is discussed.

INTRODUCTION

Molecular orientation at the air/water interface consists of the adsorption in the surface of one end or side of a molecule in preference to the other. For polar-nonpolar compounds, *e.g.*, organic paraffin derivatives containing polar groups composed of N, S, O or unsaturated carbon-carbon bonds, the polar groups will be positively adsorbed in the surface, while the nonpolar paraffin region is raised into the energetically more favorable vapor phase above the surface. The adsorption of the polar region of the molecule depends largely on the group's ability to form H-bonds with water; if the formation of these bonds is prevented or decreased, the molecular orientation may be altered[1].

Prediction of molecular orientation at the air/water interface is relatively simple for compounds like the fatty acids and alcohols, where the distribution of polar and nonpolar moieties is distinct[2]. For the structurally more complicated compounds which do not have clearly defined polar and non-polar regions, but are nevertheless surface active, the prediction of their orientation at the air/water interface is necessarily more difficult. This is generally true for the pharmacologically important compounds like the plant alkaloids where subtle changes in molecular structure have profound effects on the pharmacological activity[3], as well as their orientation at the air/water interface.

In this study, a representative group of compounds from the veratrum alkaloids[4] were examined with regard to the effect molecular structure has on the orientation these compounds assume at the air/water interface; the particular compounds were chosen because the broad variety of pharmacological activity which they exhibit[5] would permit us to examine whether a relationship exists between their pharmacological action and molecular orientation at the air/water interface.

EXPERIMENTAL

Surface tension

The drop-weight method[2] was used to measure surface tensions, and the empirical corrections of HARKINS AND BROWN[6] were applied. A stainless steel rod was cut in a lathe to 5 cm in length and 0.5 cm in diameter; a 1 mm diameter hole was bored coaxially through it. One end surface of the tube was ground plane in the lathe so that under 10 × magnification no imperfections were observed in either the rim or the surface. This tube was connected by a short piece of heavy walled rubber tubing to a syringe driven by a micrometer screw, and the entire apparatus was mounted vertically. A tared 2 ml pycnometer with a neck 0.9 cm inside diameter was used to collect the drops. Approx. 90 % of each drop was rapidly preformed, then allowed to reach full size under the force of gravity. Each drop was collected and weighed; a total of ten drops was used to obtain the average surface tension, the deviation from the mean always being less than 0.5 %. All measurements were made at 25° ± 0.5°. The value measured for water by this method was 71.95 dynes/cm compared to the reported value of 71.97 dynes/cm (see ref. 7).

Surface pressures

A Langmuir-type film balance (the Cenco hydrophil balance) was used to measure the surface pressures of the monolayer[8]. The tray was heavily coated with paraffin and the float assembly modified by using polyethylene end-loops (0.002 inch thick) and an aluminum float coated with a thin layer of teflon. Aluminum bars coated with paraffin were used as barriers.

Since the alkaloids studied do not form stable films on water even at pH's greater than 7, the following technique was devised, which permitted reproducible F-A (surface pressure–area) measurements. At each area a new monolayer was spread on water at pH 12.2 and the surface pressure exerted by the film 30 sec after deposition recorded. This time was sufficient for evaporation of the spreading solvent (benzene), but was too short for much film solution to occur. The 1- and 2-min values were also recorded at each area, and from these values the relative change of surface pressure with time was found to be independent of the initial pressure. Extrapolating these values to zero time raises by about 20 % the values of F_S (the surface pressure after 30 sec) reported here. It will be seen shortly that the significant data are the areas at which transitions in the F_S-A curves occur; these will not be significantly altered by the relatively constant and small decline in F in 30 sec in the neighborhood of the transition points. After F_S had been recorded the surface was swept clean by the barriers, and a new monolayer at a different area was prepared and measured. After a completed run the substrate was removed and the run repeated. Since contamination of the surface by the Gibbs' layer formed by alkaloid molecules dissolved in the substrate is a real problem, an adequate check on the cleanliness of the surface was to measure the surface pressure between the spreading of each new film. If a surface pressure greater than 0.1 dyne/cm was developed when the barriers decreased the length of the surface from 50 cm to 5 cm, the substrate was replaced. With these precautions, the results obtained were reproducible to within 0.1 dyne/cm. This technique, utilized exclusively in the present study, will be referred to as "static".

Veratridine, cevadine and veracevine were obtained from Professor KRAYER of the Department of Pharmacology, Harvard Medical School; the veratramine sample from Dr. O. WINTERSTEINER of the Squibb Institute for Medical Research. All the alkaloids were purified, white preparations and were employed without further purification. The drugs dissolved readily in benzene, except veracevine which was dissolved in ethanol.

Benzene (Mallinkrodt, thiophene free) was prepared free of surface active contaminants by filtering through a column of silica gel and florisil.

All aqueous solutions were prepared from water obtained from a quartz still. pH 12.2 water was prepared by adding the appropriate amount of NaOH, pH 7.2 water by adding an all sodium Sørensen phosphate buffer (1 mM).

Molecular areas

These were estimated by first constructing, with Catalin models of the atoms (scaled 1 cm = 1 Å), three dimensional models of the compounds, and then tracing the plane projection of the model in its various favored orientations onto paper. The total area of this plane projection was obtained with a planimeter, and represented the approximate surface area occupied by the drug in a particular orientation.

RESULTS

Structure

The structures of veratridine, cevadine[9], veracevine[10] and veratramine[11] are shown in Fig. 1. They are bases with p*K* values* of about 9–10 (see ref. 12). The relation between the first three compounds may be further visualized by the following esterifications:

veracevine + veratric acid = veratridine
veracevine + tiglic acid = cevadine

Surface tension

The surface activities of these compounds are shown in Fig. 2, where the surface

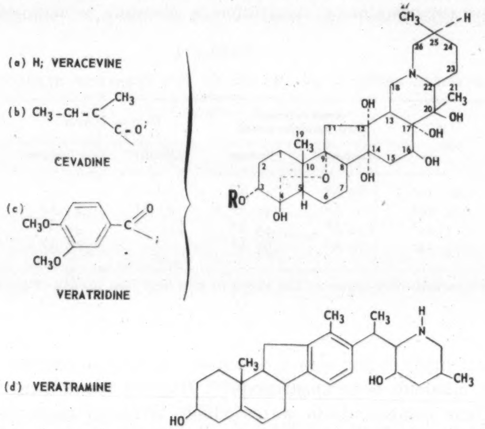
(a) H; VERACEVINE
(b) CH3-CH=C CH3 C=O
CEVADINE
(c) CH3O CH3O C=O
VERATRIDINE
R
OH
H
OH
CH3
CH3
OH
OH
OH
OH
OH
H
CH3
CH3
CH3
H N
HO
CH3
HO
(d) VERATRAMINE

Fig. 1. Structural relationships among the veratrum alkaloids. If R is replaced by (a), (b), or (c) one obtains veracevine, cevadine, or veratridine, respectively.

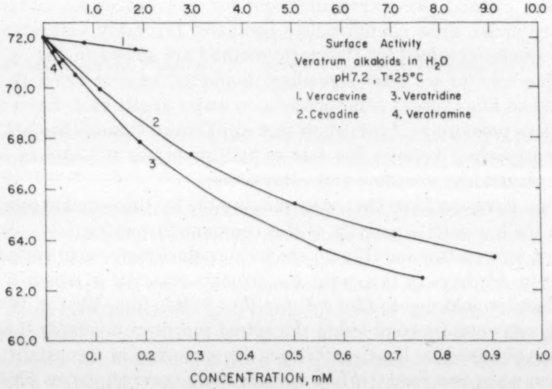

Fig. 2. The effect of lowering the surface tension, γ, of water by the veratrum alkaloids. Curve 1, concentration scale on top; curves 2, 3, and 4, concentrations listed on bottom.

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* The p*K* value for veratridine is presented here and since veracevine and cevadine also have the identical tertiary nitrogen groups we may assume their p*K* values are similar to veratridine. The p*K* value for veratramine is estimated from the value for piperidine.

tensions of solutions of the alkaloids in water at pH 7.2 are compared at various concentrations of the alkaloids. While veracevine is not very surface active, esterification of the –OH group in position 3, with either veratric acid or tiglic acid yields the highly surface active veratridine and cevadine, respectively. Veratramine is the most surface active of this group, but its limited solubility prevented measurement of surface tensions beyond the indicated concentrations. If we assume that the initial slopes of these curves are linear, then $-\Delta\gamma/\Delta C$ will be a measure of their surface activities; these values are listed in Table I, and indicate the following order for surface activity: veratramine > veratridine > cevadine ≫ veracevine.

TABLE I

PHYSICAL CHEMICAL PROPERTIES OF THE VERATRUM ALKALOIDS

Compound	$-\frac{(\Delta\gamma)^*}{(\Delta C)}$	*Areas estimated from molecular models*		*Observed areas at:*		*Probable orientations at the A/W interface H = horizontal; V = vertical*
		Horizontal	*Vertical*	*"Shoulder"*	*Film "collapse"*	
Veracevine	$0.0194 \cdot 10^4$	85 Å^2	45 Å^2	—	—	No stable film
Cevadine	$1.82 \cdot 10^4$	100 Å^2	45 Å^2	85 Å^2	42 Å^2	H, V
Veratridine	$2.12 \cdot 10^4$	130 Å^2	45 Å^2	110 Å^2	42 Å^2	H, V
Veratramine	$2.74 \cdot 10^4$	76 Å^2	35 Å^2	—	60 Å^2	H

* This value represents the mean of the slope of the first two points of each curve.

F-A curves

Previous attempts to measure *F-A* (surface pressure-molecular area) curves for these veratrum alkaloids were unsuccessful[13]. Much of the difficulty arises from the solubility of these compounds in water which, although slight, leads to film instability[14]. This is true even at elevated pH's which presumably reduce the solubility of these salts by conversion to the free base. Some of the difficulties are obviated with the static method (see EXPERIMENTAL), for *F-A* curves are obtained whose reproducibility under these circumstances compares favorably with standard techniques[3]. The results obtained by the static method are shown in Fig. 3, where F_S, the surface pressure 30 sec after spreading, is plotted against molecular areas, A, in Å^2 for alkaloid films spread from benzene on water at pH 12.2. Even though the values of surface pressure so obtained are not equilibrium values, they are still useful for relative comparison because the rate of fall of surface pressure is very nearly the same for veratridine, cevadine and veratramine.

Veracevine forms no films that were measurable by these techniques. This is in keeping with the low surface activity of this compound in water.

The curve for veratramine (Fig. 3) shows a gradual increase in surface pressure to an area of 60 Å^2; beyond this point F_S, remains constant at about 6 dynes/cm. The latter effect is analogous to film collapse for a stable film. We can better understand this phenomenon by considering the actual processes involved. Each point in the isotherm is obtained by limiting the area for spreading of a constant number of film molecules; when the water surface is completely covered, excess film molecules must be either forced above the adsorbed monolayer, or squeezed into the aqueous sub-phase. In either case no further increase in surface pressure would be expected. This area, 60 Å^2, represents the molecular area of veratramine in a state of close-packing in an orientation which is favorable for adsorption at the air/water interface.

The observed value is close to the molecular area of 76 Å^2 for veratramine in a horizontal orientation estimated from molecular models (see Table I). The –OH group in the 3-position and the piperidine nucleus are strong hydrophilic centers, each capable of forming H-bonds with water. Since these groups are at either end of the major axis of veratramine, their strong adsorption in water would necessitate a horizontal molecular orientation at the air/water interface.

The curves for cevadine and veratridine at the larger areas parallel the curve for veratramine in Fig. 3. But at about 6 dynes/cm where F_S remains constant for veratramine, "shoulders" appear in the cevadine and veratradine curves at 85 Å^2, and 110 Å^2, respectively; beyond the "shoulders" the curves rise to a limited area of 42 Å^2 and values of 9 and 10.5 dynes/cm, respectively, where F_S then remains constant.

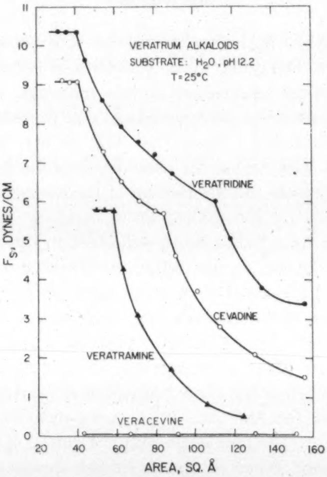

Fig. 3. F_S–A (static surface pressure-area) curves of the veratrum alkaloids on pH 12.2 water. For explanation of F_S see text.

From Table I, we see that the areas at which the "shoulders" occur approximate the areas of cevadine and veratridine estimated from models horizontally oriented in the interface, *i.e.*, 100 Å^2 and 130 Å^2 respectively; and the areas at which F_S remains constant (42 Å^2) are in good agreement with the estimated value of 45 Å^2 for the vertical orientation of these compounds with the ring N embedded in the aqueous sub-phase.

These results are consistent with a reorganization of the film molecules from a horizontal to a vertical orientation. At areas greater than the "shoulder" the molecules are horizontally oriented, much like in the veratramine film. But unlike veratramine at areas smaller than the "shoulder" a moiety of the veratridine and cevadine molecules can be raised into the vapor phase to permit an accommodation of more film molecules per unit surface area; as the areas become smaller increased numbers become vertically oriented. The vertical orientation also enhances the extent of Van der Waal's interactions between the film molecules, and this is reflected in the rise in F_S. When the vertically oriented molecules are close-packed, the area observed will be for that molecular cross-section which presents the most hydrophilic surface to the interface. That both molecules give the same cross-sectional area for their vertical orientation indicates the ring N as the most hydrophilic group in the molecule, since this group is the only one which is common to both and has the proper dimensions.

DISCUSSION

An important question arises regarding the role of -OH groups in the orientation of the molecules. Thus, if the -OH group in the 3 position in veratramine is so hydrophilic that it prevents the vertical orientation of this molecule, ought the several -OH groups distributed on veratridine and cevadine also prevent their transition from horizontal to vertical orientation? Since the -OH groups depend largely on their ability to form H-bonds with water for their hydrophilic properties, any decrease in this capacity would decrease the interaction of the molecule with water. This may arise from either a competitive intramolecular H-bonding* or by the steric blocking of the approach of water by a bulky group adjacent to the hydrophilic center. The -OH groups in positions 12, 14, 16, 17 and 20 are situated very favorably for intramolecular H-bonding and the veratric or tiglic ester group on position 3 may very well either shield or form H-bonds with the -OH group on position 4. The ester linkage itself and the lactone bridge between position 4 and 9 are much less hydrophilic than -OH[15]. The net effect is to decrease the hydrophilic properties of these groups to the extent that when the films become close-packed, additional molecules may be accommodated in the film only by a reorganization of the film where the less hydrophilic portions of the molecules may be raised into the vapor phase by pivoting around the N atom. When all the molecules are close-packed in the vertical orientation no further reorganization in the film is possible and excess molecules are forced into the sub-phase or on the surface of the film. The pressure remains constant in this case.

While many attempts have been made to relate the surface activity of a compound with its pharmacological action[16-19], they have for the most part dealt with the distribution of the chemical agent between the "biophase" and the bathing solutions. What has been largely overlooked is the possibility that molecular orientation of the chemical agent at the cell/plasma interface may also contribute to its ultimate action as a drug.

The results of the present experiments indicate that the veratrum alkaloids, though they are structurally similar, exhibit contrasting behavior at the air/water interface. Veracevine is only slightly surface active in water; veratridine, cevadine, and veratramine are very surface active, with the first two capable of orienting horizontally as well as vertically, while veratramine shows only a horizontal orientation at the air/water interface.

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* A striking example of this effect is found by comparing the solubilities of the *o*- and *m*-hydroxybenzoic acids in water, the former being 0.18 and the latter 0.92 g/100 ml at 20°. (Handbook of Chemistry and Physics, 38th Edition, Chemical Rubber Publishing Co.) Intramolecular H-bonding between the -COOH and -OH groups reduces the solubility of the *ortho* compound while intermolecular H-bonds between -OH and -COOH and water enhances the solubility of the *meta compound*.

Pharmacologically, we find they also exhibit diverse behavior[5]. Veracevine is inactive*, veratridine and cevadine are "labilizers" or "unstabilizers"[20], and veratramine is an antagonist of veratridine and cevadine[21]. Little is known of the actual mechanism for action of these drugs, but their seemingly parallel physical and pharmacological properties suggest that the orientation of these compounds at the cell surface may at least be partially responsible for their pharmacological activity.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Drs. A. M. **Shanes** and W. A. **Zisman** for the advice and encouragement which they so graciously contributed.

REFERENCES

[1] N. K. Adam, *The Physics and Chemistry of Surfaces*, 3rd Edition, Oxford University Press, 1941, p. 77.
[2] W. D. Harkins, *The Physical Chemistry of Surface Films*, Rheinhold Publishing Corp., 1952.
[3] L. S. Goodman and A. Gilman, *The Pharmacological Basis of Therapeutics*, 2nd Edition, The Macmillan Company, New York, 1955.
[4] K. J. Morgan and J. A. Barltrop, *Quart. Revs.* (*London*), 12 (1958) 34.
[5] O. Krayer and G. H. Acheson, *Physiol. Revs.*, 26 (1946) 383.
[6] W. D. Harkins and F. E. Brown, *J. Am. Chem. Soc.*, 41 (1919) 499.
[7] *Lange's Handbook of Chemistry*, 9th Edition, Handbook Publishers, Inc., Sandusky, Ohio, 1956.
[8] N. K. Adam, *The Physics and Chemistry of Surfaces*, 3rd Edition, Oxford University Press, 1941, p. 27–31.
[9] M.V. Mijovic, E. Sundt, E. Kyzburz, O. Jeger and V. Prelog, *Helv. Chim. Acta*, 38 (1955) 231.
[10] K. J. Morgan and J. A. Barltrop, *Quart. Revs.* (*London*), 12 (1958) 46.
[11] V. Prelog and O. Jeger, in H. F. Manske and H. L. Holmes, *The Alkaloids*, Vol. 3, Academic Press, New York, 1953, p. 282.
[12] I. M. Kolthoff, *Biochem. Z.*, 162 (1925) 289.
[13] A. Rothen and L. C. Craig, *J. Am. Chem. Soc.*, 65 (1943) 1102.
[14] C. G. Lyons and E. K. Rideal, *Proc. Roy. Soc. London, A*, 124 (1929) 344.
[15] O. Kamm, *Qualitative Organic Analysis*, John Wiley and Sons, New York, 2nd Edition, 1932, Ch. 2.
[16] I. Traube, *Arch. ges. Physiol., Pflüger's*, 105 (1904) 541.
[17] O. Warburg, *Arch. ges. Physiol., Pflüger's*, 144 (1940) 465.
[18] L. E. Tammelin and N. Löfgren, *Acta Chem. Scand.*, 1 (1947) 871.
[19] J. C. Skou, *Acta Pharmacol. Toxicol.*, 10 (1954) 325.
[20] A. M. Shanes, *J. Pharmacol. Exptl. Therap.*, 105 (1952) 216.
[21] O. Krayer and H. W. George, *J. Pharmacol. Exptl. Therap.*, 103 (1951) 249.

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* The low surface activity and pharmacological inactivity (see ref. 5) of veracevine would indicate for the veratrum alkaloids at least, that surface or interfacial activity is necessary to concentrate the drug at or near its site of action.

STUDIES ON ENZYME RADIOSENSITIVITY

I. THE EFFECT OF BICARBONATE ON LIVER ALCOHOL DEHYDROGENASE

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SUMMARY

When dilute aqueous solutions of liver alcohol dehydrogenase were exposed to X-irradiation, there was much more inactivation in the presence of bicarbonate buffer than in phosphate buffer or in distilled water. The effect was not due to the action of any stable intermediates produced by irradiation of bicarbonate buffer, nor does it seem likely that the enzyme is converted by irradiation to some long-lived metastable state whose destruction is precipitated by the presence of bicarbonate. Enzyme and bicarbonate must be present simultaneously for the effect to be observed. Several other enzymes have been investigated in this regard, and possible mechanisms discussed.

INTRODUCTION

A wide variety of data are available in the literature concerning the inactivation of purified enzymes by ionizing radiations. DALE[1-4] and BARRON[5,6] have laid important groundwork in this area. Other such studies have been carried out by MCDONALD[7-9] with trypsin and chymotrypsin, by SETLOW AND DOYLE[10], BONET-MAURY AND PATTI[11] and SUTTON[12] with catalase, by ANDERSON[13] with pepsin, by BABIN[14] with papain, and by ROBINSON, PHILLIPS AND DILG[15] with a variety of enzymes. Uniformly, these workers have reported that added solutes either protected the enzyme from irradiation damage or else had no effect, except in the case of dissolved oxygen which increased the radiosensitivity of several sulfhydryl enzymes[16]. It was therefore of great interest in the present study to observe the striking enhancement of the radiosensitivity of liver alcohol dehydrogenase in the presence of bicarbonate buffers.

EXPERIMENTAL

Enzyme

Crystalline horse liver alcohol dehydrogenase (lot No. 5521) was obtained from the Worthington Biochemical Corporation. When assayed by the method of BONNICHSEN *et al.*[17] this was found to be about 20% active enzyme. For subsequent assays DPN reduction was followed between 0.5 and 1.5 min rather than for 3 min as in the method of BONNICHSEN, and Tris buffer was used rather than glycine buffer.

Irradiation

250 kV X-rays were used. The enzyme samples were dissolved in 1 ml of solution and exposed in 5 ml Pyrex glass beakers using a Plexiglas holder for reproducible positioning in the X-ray beam. For irradiation in gases other than air a sealed holder was used. This was evacuated for 10 min with a water aspirator, flushed for 5 min with nitrogen or helium and the evacuation and flushing repeated before sealing off the system containing the inert gas at atmospheric pressure. Ferrous-ferric dosimetry[18] was used routinely and (since the dosimeter yield is dependent on oxygen saturation) indicated thorough removal of oxygen by this procedure.

The enzyme concentration was 100 μg protein/ml of solution. Following the irradiation period, 0.1 ml aliquots were withdrawn from each sample for assay. Controls were shielded with lead and kept in the X-ray room during exposure of the other samples.

Comparison of buffers

Enzyme was irradiated at a dose of 7–12 Krep in phosphate and bicarbonate buffers at various pH values but all at 0.1 *M* concentration. The inactivation date are plotted in Fig. 1. In the range between pH 8 and 9, where it was possible to overlap these buffers, the difference between them is apparent. This difference was clearly not a pH effect, but, rather, was dependent on the specific anions. Other buffers could not conveniently be used in this range because they strongly protected the enzyme against X-irradiation. For example, with 0.1 *M* borate buffer an X-ray dose of 20 Krep was required to achieve inactivation similar to 10 Krep with phosphate buffer. Some data with borate are given in Fig. 2 and indicate increased protection at decreased pH values. This may indicate that boric acid is more effective as a protector than is borate anion. These observations, however, have not been studied further.

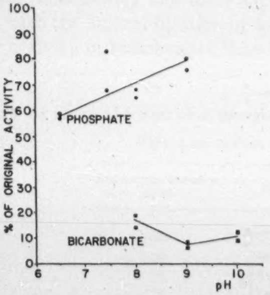


Fig. 1. Per cent of activity of liver alcohol dehydrogenase remaining after 12 Krep X-irradiation in 0.1 *M* phosphate or bicarbonate at various pH values. Each sample had 100 μg protein dissolved in 1 ml of buffer.

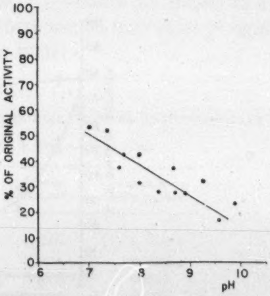


Fig. 2. Per cent of activity of liver alcohol dehydrogenase remaining after 30 Krep X-irradiation in 0.1 *M* sodium borate buffer at various pH values. Each sample had 100 μg protein dissolved in 1 ml of buffer.

Assay by the reverse reaction

A comparison between phosphate and bicarbonate buffers was also made by using the reverse reaction:

$$\text{acetaldehyde} + \text{DPNH} \longrightarrow \text{ethanol} + \text{DPN}$$

to assay enzyme activity. For this method of assay the following additions were made to a 1 cm Beckman cuvette: 0.50 ml Tris buffer 0.5 *M*, pH 8.5, 2.25 ml water, 0.05 ml 0.005 *M* DPNH, 0.10 ml 10 % v/v acetaldehyde.

Enzyme was added (0.1 ml) and the change in absorption between 1 and 2 min read at 340 mμ. Because of the rapidity of this reverse reaction the enzyme samples were diluted by adding 2 ml of water to the 1 ml samples before withdrawing 0.1-ml aliquots. The results of this assay were entirely comparable with the data obtained from the forward reaction (Fig. 1) so that the bicarbonate sensitization applies to the inactivation of the catalytic activity regardless of the direction of the reaction catalyzed.

Inertness of phosphate

The data of Fig. 1 without further evidence could be interpreted as resulting either from protection by phosphate or sensitization by bicarbonate. It was found that at pH 6.5 irradiation in 0.1 *M* phosphate gave just as much inactivation as irradiation in distilled water at this pH. The results of more conclusive experiments are given in Fig. 3 and Table I. Fig. 3 shows that as the concentration of bicarbonate buffer was reduced to zero while keeping the pH constant at 9, the amount of enzyme inactivated approached the value observed in phosphate buffer. Varying the phosphate concentration had a negligible effect on enzyme radiosensitivity. If phosphate were protective and bicarbonate inert, it would be expected that the curve for the latter would be a straight line cutting the ordinate at the same point as the curve for phosphate extrapolated to zero phosphate concentration. The activity for phosphate would then rise with increasing phosphate concentration.

Further corroboration for the inertness of phosphate is shown in Table I. Here, activity is given for the two buffers separately and mixed. All activities were the

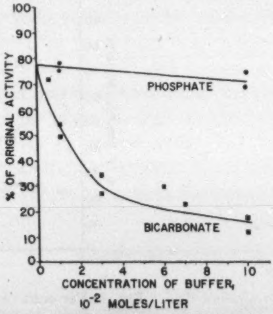


Fig. 3. Inactivation of liver alcohol dehydrogenase in phosphate and bicarbonate buffers at different concentrations but all at pH 9. X-ray dose: 12 Krep. Each sample had 100 μg protein in 1 ml of buffer.

TABLE I

INACTIVATION OF LIVER ALCOHOL DEHYDROGENASE BY X-IRRADIATION IN PHOSPHATE AND BICARBONATE BUFFER SEPARATELY AND MIXED

X-ray dose 12 Krep. Enzyme concentration 100 μg/ml. Activity values represent the O.D. change in 1 min at 340 mμ.

	Buffer used (0.1 M, pH 9)	*Activity*
X-rayed Samples	Bicarbonate	0.015 0.008
	Phosphate	0.049 0.043
	Mixed bicarbonate + phosphate	0.015 0.010
Non-X-rayed Controls	Bicarbonate	0.055 0.056
	Phosphate	0.056 0.061
	Mixed bicarbonate + phosphate	0.056 0.057

same in non-irradiated samples, whereas in the mixed buffer there was the same amount of inactivation as in bicarbonate alone, indicating no protection by the phosphate; or, stated in another way, the bicarbonate produced its sensitization effect even in the presence of phosphate.

Effect of bicarbonate on non-irradiated enzyme

The data presented above have shown that, at the concentration of enzyme and buffer normally used, bicarbonate had no inhibitory effect on the enzyme. The possibility remained that in a partially radiation-inactivated (*i.e.* more dilute) enzyme solution, inhibition by bicarbonate could produce an effect which would be interpreted as enhancement of radiosensitivity. To test this possibility several dilutions of enzyme were prepared in 0.1 M, pH 9.0 bicarbonate and phosphate buffers. These were assayed immediately and after a period of time. The results are shown in Table II. Even with the highest dilution of enzyme used there was no indication of significantly lower activity in bicarbonate than in phosphate buffer.

TABLE II

ACTIVITY OF LIVER ALCOHOL DEHYDROGENASE DILUTED IN BICARBONATE AND PHOSPHATE BUFFERS

Values represent O.D. change in 1 min at 340 mμ.

Enzyme concentration, μg/ml	*Buffer used*	*Activity immediately*	*Activity after 30 min*
100	Phosphate	0.016	0.013
100	Bicarbonate	0.021	0.013
75	Phosphate	0.034	0.037
75	Bicarbonate	0.035	0.034
50	Phosphate	0.044	0.049
50	Bicarbonate	0.043	0.042
25	Phosphate	0.061	0.062
25	Bicarbonate	0.056	0.053

Irradiation of enzyme and bicarbonate separately

To determine whether stable products formed on irradiation of bicarbonate solutions were responsible for inactivating the enzyme, solutions of 0.1 M, pH 9.0 phosphate and bicarbonate buffers were irradiated and then used for dilution of the enzyme in the same way as non-irradiated buffer solutions. Results of such an assay are given in Table III. There was no significant inactivation of enzyme by either of the irradiated solutions.

TABLE III

ADDITION OF IRRADIATED AND NON-IRRADIATED BUFFERS (0.1 M, pH 9) TO NON-IRRADIATED ENZYME

Final enzyme concentration 100 μg/ml. Buffer (0.9 ml) irradiated with 12 Krep and added immediately to 0.1 ml concentrated enzyme. Activity values represent change in O.D. in 1 min at 340 mμ.

	Buffer used	*Activity*
Irradiated Buffer	Phosphate	0.056
	Phosphate	0.064
	Bicarbonate	0.051
	Bicarbonate	0.053
Non-irradiated Buffer	Phosphate	0.059
	Phosphate	0.059
	Bicarbonate	0.056
	Bicarbonate	0.065
Enzyme irradiated in bicarbonate		0.010
Buffer	—	0.006

To see whether the enzyme might somehow be "weakened" by irradiation and thereby made susceptible to inactivation by bicarbonate, solutions of enzymes were irradiated in phosphate buffer and immediately mixed with bicarbonate buffer to give a final solution of pH 9.0 and 0.1 M in phosphate and bicarbonate. This was then assayed as usual, and typical results are given in Table IV. There was no indication that the suggested mechanism was operative.

TABLE IV

The enzyme was irradiated in phosphate buffer, pH 9, 0.125 M, at 12 Krep and then mixed with bicarbonate buffer or water to give final concentration of 0.1 M phosphate and bicarbonate before assaying. Enzyme concentration 100 μg/ml. Activity figures represent O.D. change in 1 min at 340 mμ.

	Diluted with water	*Diluted with bicarbonate*
Enzyme in phosphate, irradiated	0.045	0.050
	0.041	0.046
Enzyme in phosphate, control	0.060	0.060
	0.066	0.060
Enzyme irradiated in mixed buffer	0.024	—
	0.018	—

The oxygen effect

In an attempt to gain more insight as to possible mechanisms of the bicarbonate effect, enzyme solutions in phosphate and bicarbonate buffers (0.1 *M*, pH 9.0) were irradiated in atmospheres of nitrogen and helium for comparison with results obtained in air. Results of such experiments are given in Fig. 4. At the X-ray dose used, enzyme dissolved in phosphate buffer was negligibly inactivated whether in air or in an inert atmosphere. Enzyme dissolved in bicarbonate buffer, however, showed considerably more inactivation in the presence of oxygen; but, in any case, the increasing sensitivity of enzyme with increasing bicarbonate concentration was maintained regardless of the atmosphere used. Fig. 4 also shows that there was a very gradual loss of enzyme activity after irradiation whether in air or in nitrogen. Since this delayed inactivation appeared not to be accelerated when irradiation was carried out in air, it is presumably a simple thermal process and not the result of inactivation by hydrogen peroxide in the solution.

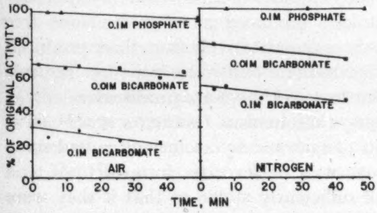


Fig. 4. A comparison of enzyme inactivation in nitrogen and air with three different buffer systems. The time coordinate represents time interval after the end of the X-ray period. X-ray dose: 7 Krep. Enzyme concentration, 100 μg/ml.

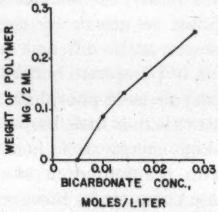


Fig. 5. Polymethylmethacrylate formed *vs.* concentration of bicarbonate in pH 9 aqueous solution. 2 ml of 0.1 *M* monomer irradiated with 35 Krep in a helium atmosphere.

A methacrylate model system

Since DAINTON[19], and others have shown that the polymerization of various acrylic monomers in aqueous solution is catalyzed by radiation-produced hydroxyl radicals, we have investigated the effect of bicarbonate on such a system to see whether it would increase the amount or speed of polymerization.

Methyl methacrylate was dissolved in deaerated water with various concentrations of bicarbonate buffer at pH 9 to give a 0.1 *M* solution of monomer. 2-ml samples were irradiated in a helium atmosphere at a dose of approximately 35 Kreps. Coagulated polymer was then separated by centrifuging, washed several times with water and determined quantitatively using a colorimetric, chromic acid oxidation method. Results of a typical experiment are plotted in Fig. 5. Polymer formation was definitely increased with increasing bicarbonate concentrations, although this was observed over a much narrower range of concentration than was found for the enzyme sensitization.

Other enzymes

Preliminary experiments have been carried out to see whether the bicarbonate sensitization effect applies to other enzymes as well as to liver alcohol dehydrogenase. With yeast alcohol dehydrogenase there was an apparent sensitization if samples were assayed several minutes after irradiation. This, however, was found to be an artifact which resulted from the fact that dilute solutions of yeast alcohol dehydrogenase were more rapidly inactivated in bicarbonate than in phosphate buffer, regardless of irradiation. Other enzymes which were examined with negative results were yeast hexokinase, muscle aldolase, and muscle lactic dehydrogenase.

DISCUSSION

The data presented leave no doubt as to the validity of the bicarbonate sensitization effect. Clearly, liver alcohol dehydrogenase is much more sensitive to the action of X-irradiation in the presence of bicarbonate buffer than in its absence.

In general terms, three possible mechanisms can be adduced to explain the data: (a) Irradiation of bicarbonate solutions produces some highly reactive and unstable intermediate which is especially effective in attacking the enzyme. (b) When dissolved in bicarbonate buffer, the enzyme assumes a configuration which renders it particularly susceptible to attack by the free radicals produced in solution. These free radicals, however, are no different qualitatively or quantitatively from those produced by irradiation of phosphate buffers. (c) In bicarbonate buffer the same free radicals attack the enzyme as in phosphate buffer, but more of them are produced.

At present there is little basis for choosing a mechanism. GARRISON *et al.*[20], have shown that high energy alpha bombardment of carbonic acid solutions caused some reduction with the formation of small quantities of formate, formaldehyde and oxalate. These compounds, however, are all sufficiently stable so that if they were responsible for inactivating the enzyme, addition of irradiated buffer solution to the non-irradiated enzyme should have the same result as irradiation of the two simultaneously. In addition, judging from the data of GARRISON *et al.*, the concentrations of these substances which would have been formed in our system are vanishingly small.

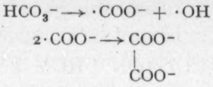
If mechanism (a) applies, the required intermediate must be unstable and most likely a free radical. Peroxycarbonates are known to be produced by the reaction of hydrogen peroxide with alkali carbonates. These are strong oxidizing and reducing agents which presumably could be formed by irradiation of carbonate solutions in the presence of oxygen. However, they are not unstable enough to account for our data; and, more important, they presumably could not be formed in the absence of oxygen, where the bicarbonate sensitization was still observed.

Concerning mechanism (b), likewise, little can be said. However, the fact that bicarbonate strongly inhibits yeast alcohol dehydrogenase irrespective of irradiation may be taken as suggesting that it does have some effect on enzyme protein. Clarification of this inhibitory effect with the yeast enzyme might be helpful in understanding the mechanism of bicarbonate sensitization of the liver alcohol dehydrogenase.

At the present, a combination of mechanisms (b) and (c) seems best to explain the data. While water is decomposed into free radicals by radiation as follows:

$$H_2O \longrightarrow \cdot H + \cdot OH$$

the reverse reaction also readily occurs to limit the amount of hydroxyl radical present if nothing is available for the hydrogen to react with. As shown by the work of GARRISON *et al.*[20] irradiated bicarbonate forms oxalate presumably as follows:



$$HCO_3^- \longrightarrow \cdot COO^- + \cdot OH$$

$$2\cdot COO^- \longrightarrow \begin{array}{c} COO^- \\ | \\ COO^- \end{array}$$

so that as the carboxyl radicals combine with each other to form oxalate, the hydroxyl radical is left without a partner and could reach greater concentrations than in the irradiation of pure water.

The methacrylate polymerization reaction, known to be catalyzed by hydroxyl radicals confirms the above hypothesis since polymerization was increased by increasing the bicarbonate concentration.

An increased concentration of hydroxyl radicals is alone insufficient to account for the bicarbonate effect with liver alcohol dehydrogenase, however, since other enzymes which are also inactivated by hydroxyl radicals were not found to be sensitized by bicarbonate. It seems reasonable to invoke mechanism (b) to explain this difference and to suggest that with this particular enzyme the bicarbonate may be more closely associated with the protein than with the other enzymes tested so that not only is the concentration of hydroxyl radicals increased but it is increased in the immediate vicinity of the enzyme groups to be inactivated. Carbon dioxide is known to form more or less tight complexes with the free amino groups of certain proteins[21], and such complexes might play a role here. At least their existence shows that bicarbonate may be closely associated with enzyme protein.

ACKNOWLEDGEMENT

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REFERENCES

[1] W. M. DALE, *Biochem. J.*, 34 (1940) 1367.

[2] W. M. DALE, *Biochem. J.*, 36 (1942) 80.

[3] W. M. DALE, J. V. DAVIES AND W. J. MEREDITH, *Brit. J. Cancer*, 3 (1949) 31.

[4] W. M. DALE, in A. HOLLAENDER, *Radiation Biology*, Vol. 1, Part 1, McGraw-Hill, N.Y., 1954, p. 255.

[5] E. S. G. BARRON, in A. HOLLAENDER, *Radiation Biology*, Vol. 1, Part 1, McGraw-Hill, N.Y., 1954, p. 283.

[6] E. S. G. BARRON AND P. JOHNSON, *Arch. Biochem. Biophys.*, 48 (1954) 149.

[7] M. R. McDONALD, *Trans. Faraday Soc.*, 49 (1953) 333.

[8] M. R. McDONALD AND E. C. MOORE, *Radiation Research*, 2 (1955) 426.

[9] E. C. MOORE AND M. R. McDONALD, *Radiation Research*, 3 (1955) 38.

[10] R. B. SETLOW AND B. DOYLE, *Arch. Biochem. Biophys.*, 46 (1953) 46.

[11] P. BONET-MAURY AND F. PATTI, *J. chim. phys.*, 52 (1955) 617.

[12] H. C. SUTTON, *Biochem. J.*, 64 (1956) 447, 456.

[13] R. S. ANDERSON, *Trans. Faraday Soc.*, 49 (1953) 333.

[14] R. BABIN, *Bull. soc. pharm. Bordeaux*, 93 (1955) 35; *Chem. Abstr.*, 50, 8759.

[15] T. ROBINSON, A. W. PHILLIPS AND SHIRLEY DILG, *Abstracts of Papers, American Chemical Society, September* 1957, 1 C.

[16] E. S. G. BARRON, *Symposium on Radiobiology*, Wiley, N.Y., 1952, p. 216.

[17] R. K. BONNICHSEN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, p. 495, Academic Press, N.Y., 1955.

[18] J. WEISS, *Nucleonics*, 10 (1952) 28.

[19] F. S. DAINTON, *Nature*, 160 (1947) 268.

[20] W. M. GARRISON, D. C. MORRISON, J. G. HAMILTON, A. A. BENSON AND M. CALVIN, *Science*, 114 (1951) 416.

[21] G. GIUSTINA, E. MILLA AND R. MARGARIA, *Giorn. biochim.*, 2 (1953) 357.

TRANSFER OF PILIATION FROM *ESCHERICHIA COLI* TO *SALMONELLA TYPHOSA* BY GENETIC RECOMBINATION

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SUMMARY

The ability to produce pili, the hair-like, non-flagellar appendages of bacteria, has been transferred from *Escherichia coli* to *Salmonella typhosa* by genetic recombination. The piliated *E. coli* donor was mixed with the permanently non-piliated *S. typhosa* recipient on solid medium which could not support the growth of either. Recombinant forms were selected in the presence of various sugars which the *S. typhosa* parent could not ferment. Hybrid forms fermenting xylose or rhamnose were, with few exceptions, piliated. Since the *S. typhosa* parent was never observed to produce a piliated form spontaneously, the occurrence of pili on hybrids which retained most of the biochemical and all of the antigenic properties of *S. typhosa* must have been due to genetic transfer. Furthermore, the antigenicity of the transferred pili was determined by the *E. coli* donor.

The piliated *S. typhosa* hybrids, which also possessed the Vi antigen, spontaneously produced two other, more stable forms, usually at a high rate. One of these lacked pili and the other lacked the Vi antigen. This rapid breakdown was probably due to the segregation of diploid heterozygotes. An analysis of the segregants showed the piliation character to be linked to arabinose fermentation.

INTRODUCTION

Pili are non-flagellar, hair-like appendages, unrelated to motility, found growing from the surface of many different species of bacteria. They are thinner, straighter, and more numerous than flagella and have a uniform diameter of 80–100 Å. Many piliated strains of bacteria can exist in two forms, the completely piliated (P+) or the completely non-piliated (P—), and these strains may change from one form to the other at a high rate[1-3].

The P+ form of *Escherichia coli* B–L(E), studied by Brinton[3], however, spontaneously gives rise to stable P— forms which do not revert to P+. Presumably, other strains not having pili have been isolated as stable P— forms from nature. A stable P— strain, thus, could not produce pili unless it were to change spontaneously

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(rare mutation to P+), or unless it could be made P+ by a process of genetic transfer such as bacterial recombination[4] or transduction[5].

The present account is a report on the transfer of the P+ character from a high frequency of recombination (Hfr) strain of *E. coli* K-12 (see ref. 6), to a stable P— strain of *Salmonella typhosa* as a result of genetic recombination[7]. The serological cross-reaction with *E. coli* in certain hybrids from matings between donor (Hfr, P+) *E. coli* and recipient (F— P—) *S. typhosa* observed by BARON *et al.*[8] is explained by the acquisition of pili (pilial antigen) by the *Salmonella* hybrids (F—, P+).

MATERIALS AND METHODS

Bacterial strains

The Hfr parent was the W1895 strain of *E. coli* K-12 (see ref. 6), which was able to ferment the sugars, lactose (Lac+), L-arabinose (Ara+), D-xylose (Xyl+), L-rhamnose (Rha+), L-fucose (Fuc+) when received from Dr. P. D. SKAAR, and D-arabinose (D-Ara+) following selection of a mutant clone able to utilize this sugar. An F— strain (643L+) of *S. typhosa* was isolated from the F° strain 643 by BARON *et al.*[9] as a Lac+ hybrid from an initial mating with W1895 and used as the recipient strain in further crosses. Strain 643L+ was able to utilize lactose as a result of the initial mating, but like the parent strain 643, still required cystine (cys—) and tryptophan (try—) and was unable to utilize any other of the above sugars. It still produced the somatic (O) antigens 9, 12, the flagellar (H) antigen "d", and the Vi antigen, components characteristic of *S. typhosa*. In common with all strains of *S. typhosa* tested, it did not cross-react with antiserum against the *E. coli* K-12 strain.

Media

0.8 % Difco nutrient broth plus 0.5 % sodium chloride was used for both liquid and solid media for purposes of routine transfer or cultivation. Plates and slant or stab tubes were prepared by the addition of 2 % agar to the liquid media.

Minimal medium was made up by mixing separately autoclaved Difco Noble Agar (14 g/400 ml H_2O); physiological saline, 300 ml; KH_2PO_4, 40 ml of 60 g/l; K_2HPO_4, 40 ml of 140 g/l; $(NH_4)_2SO_4$, 40 ml of 20 g/l; $MgSO_4$, 4 ml of 20 g/l and 10 ml of a 20 % solution of the appropriate carbohydrate previously sterilized by filtration. To this medium was added 10 ml of 0.4 % L-cystine and 10 ml of 0.4 % L-tryptophan, to satisfy the nutritional requirements of the *S. typhosa* recipient organism.

Special Eosin–Methylene Blue agar without sugars (Difco, BBL), to which was added 0.4 % of the appropriate sugar (Pfanstiehl) was employed. Phenol Red Broth (Difco) plus 1 % carbohydrate was used for fermentation tests.

Recombination techniques

The parental strains, *E. coli* W1895 and *S. typhosa* 643L+ were centrifuged from 18 h nutrient broth cultures and washed 3 times with saline. 0.1 ml containing $1 \cdot 10^7$ cells of W1895 was spread together with 0.1 ml containing $2–5 \cdot 10^8$ cells of 643L+ on plates of minimal agar containing cystine (cys), tryptophan (try), and either L-arabinose, D-xylose, L-rhamnose, L-fucose, or D-arabinose as the selective carbohydrate. The *E. coli* parent, W1895, required methionine (M—), while the *S. typhosa* hybrid 643L+, used as the recipient in these crosses, could not utilize L-arabinose or the other carbohydrates listed. M+Ara+, M+Xyl+, etc. progeny can be detected only on addition of cystine and tryptophan which fulfill the nutritional requirements of the recipient parent. Recombinants were purified by streaking on EMB agar containing the appropriate carbohydrate. In cases of ambiguity, suspect colonies were restreaked further on this medium to establish purity and stability.

Bacterial agglutination

To fourfold serial dilutions of rabbit antisera in saline were added about $5 \cdot 10^8$ bacteria per ml. After 1 h at room temperature (25–30°) the tubes were observed by oblique lighting under low magnification. The presence of visible agglutination was taken as a positive reaction. The procedures described by EDWARDS AND EWING for slide agglutination tests also were employed. Cultures were examined simultaneously in saline and tested with normal serum at the same dilution as that used for the antiserum.

Electron microscopy

Cultures were grown in aerated liquid medium to a concentration of about $3 \cdot 10^8$ cells/ml, fixed for 1 h by the addition of formaldehyde to a concentration of 2 %, and dialyzed against several changes of distilled water to remove inorganic salts. A small drop of the cell suspension was placed on a collodion film overlying a microscope grid and allowed to air dry. Specimens were shadowed with platinum and observed in an RCA EMB microscope. Some cultures were centrifuged and washed twice with distilled water before fixing and drying. This procedure resulted in a cleaner background.

Hemagglutination

Cultures to be tested for their capacity to agglutinate chicken red blood cells were diluted and spread on the surface of nutrient agar plates and incubated at 37° for 18 h. About one hundred individual colonies were combined and suspended in 1 ml of physiological saline solution. Two-fold serial dilutions were made in saline in 5-ml glass tubes. Red blood cells were centrifuged from preparations of heparinized chicken blood and washed 3 times with saline. These were added to each tube to a concentration of about 10^7 cells/ml, and after 3 h at 4° the agglutination test was read. With positive hemagglutination a diffuse sediment appears at the bottom of the tube, while lack of hemagglutination is shown by a sharply defined red button.

RESULTS

Initial observations

When the *E. coli* Hfr was crossed with the *S. typhosa* hybrid, 643L+, the number of recombinants obtained was found to vary depending on the sugar used to detect the progeny. It has been observed also that recombinants selected on plates containing xylose or rhamnose, with few exceptions, were highly unstable forms[8]. These forms could not be purified as stable positives for the sugar on which they had been selected. Two principle types of distinguishable colonies, however, could be observed by means of oblique lighting when a single positive clone from EMB agar was restreaked on nutrient agar: a small, dense colony which was shown to possess the

Vi, O, and H antigens of *S. typhosa* and which cross-reacted with antiserum prepared against living *E. coli* K-12, and a larger, more translucent form, typical of *S. typhosa* 643L+ (non-reactive with *E. coli* antisera but reacting with *S. typhosa* Vi, O, and H antisera). Further restreaking of the dense form gave rise almost entirely to stable translucent colonies, usually within a few subcultures. The dense form was so unstable that the translucent colonial type often arose as a sector within an originally dense colony.

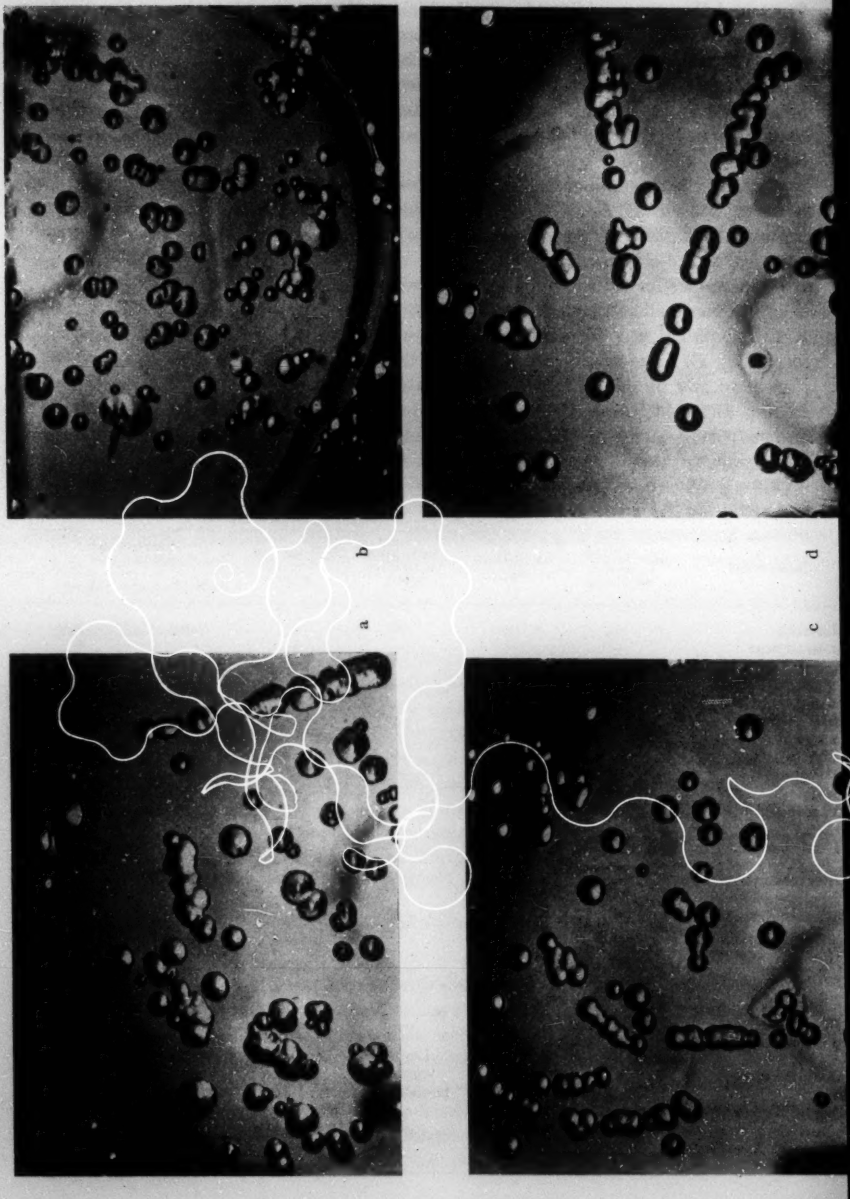
One of the recombinants, strain X30D, selected for the ability to utilize xylose, however, was a small, dense colonial form which remained relatively stable, although, occasionally it did give rise to a considerably larger, more translucent variant, X30T, and also, to a slightly smaller form, X30P.

The properties of these variants which were derived from the hybrid X30D were examined in detail and are compared with those of the parents in Table I. The *E. coli* K-12 Hfr parent was composed of two colonial types, a smooth form and a rough form (Fig. 1). Since no difference between these two types was ever observed with respect to any of the characteristics in Table I, they are both listed in the same column. Both the *S. typhosa* 643 parent and the hybrid *S. typhosa* 643L+ exhibited one main type of colony form which was rather large and glistening when viewed

TABLE I

CHARACTERISTICS OF PARENTAL AND HYBRID STRAINS

Characteristics	*Male parent E. coli K-12 Hfr W1895*	*S. typhosa 643*	*Female parent S. typhosa 643L+*	*Hybrid selected on xylose X30D*	*Segregants from hybrid X30D*	
					X30T	*X30P*
Lactose	+	—	+	+	+	+
L-arabinose	+	—	—	+	—	+
D-xylose	+	—	—	+	+	+
L-rhamnose	+	—	—	+	+	+
L-fucose	+	—	—	—	—	—
D-arabinose	+	—	—	—	—	—
L-cystine	+	—	—	—	—	—
L-tryptophan	+	—	—	—	—	—
Methionine	—	+	+	+	—	—
Indol	+	—	—	+	+	+
Vi antigen	—	+	+	+	+	—
O antigen 9, 12	—	+	+	+	+	+
H antigen d:-	—	+	+	+	+	+
Electron microscopy	Piliated	Non-piliated	Non-piliated	Piliated	Non-piliated	Piliated
Agglutination with anti-pili serum from *E. coli* B-L(E)	+	—	—	+	—	+
Agglutination with antiserum against live *E. coli* K-12, Hfr, W1895	+	—	—	+	—	+
Hemagglutination	+	—	—	+	—	+
Colonies	Large rough and smooth	Large, smooth translucent	Large, smooth translucent	Medium, dense	Large, smooth translucent	Small, dense
Agglutination with anti-serum against hybrid X30D	+	+	+	+	+	+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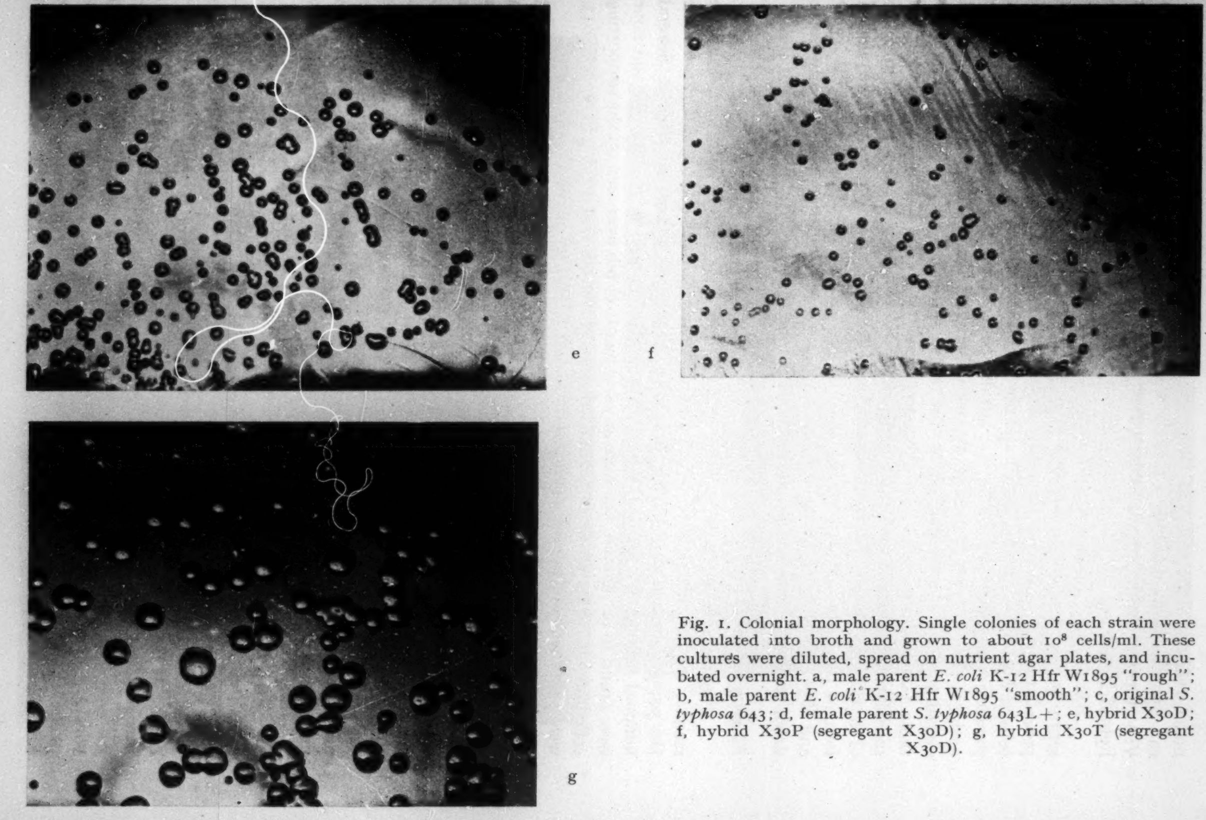


Fig. 1. Colonial morphology. Single colonies of each strain were inoculated into broth and grown to about 10^8 cells/ml. These cultures were diluted, spread on nutrient agar plates, and incubated overnight. a, male parent *E. coli* K-12 Hfr W1895 "rough"; b, male parent *E. coli* K-12 Hfr W1895 "smooth"; c, original *S. typhosa* 643; d, female parent *S. typhosa* 643L+; e, hybrid X30D; f, hybrid X30P (segregant X30D); g, hybrid X30T (segregant X30D).

under reflected light (Fig. 1). However, both 643 and 643L+ when subcultured can spontaneously produce a colonial variant which has lost the Vi antigen. The Vi+ form of 643L+ was used as the parent in the cross which produced the hybrid X30D.

BARON *et al.*[8] had observed a cross-reaction between X30D or X30P with antiserum prepared against living cells of *E. coli* K-12, by slide and tube agglutination procedures. X30T did not show this cross-reaction. An antiserum prepared against cells of *E. coli* K-12 boiled for 1 h did not agglutinate either X30D, X30P, or X30T, showing that the cross-reaction was not due to the transfer of a heat-stable somatic antigen. Other results in this study, using serum immobilization procedures, had served to eliminate the possibility of a transfer of flagellar antigen from *E. coli* to these *Salmonella* hybrids. The likelihood that a transfer of pili was involved was considered, because of the known antigenicity of pili and the remarkable resemblance of the colonial variation of the cross-reacting *Salmonella* hybrids to that of the piliated form of *E. coli* B–L(E) (see ref. 3). Both *E. coli* B–L (E) P+ and the hybrids X30D and X30P have small, dense colonies which upon sub-culture at 37° rapidly break down to larger, more translucent colonies.

Electron microscopy

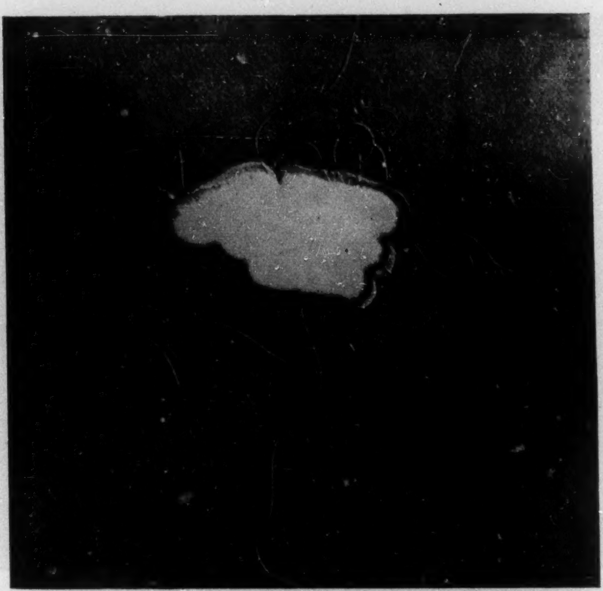
These studies were undertaken in order to detect visually and in a definitive manner the presence or absence of pili. Cells from a broth culture of the *E. coli* K-12 Hfr parent, examined in the electron microscope, were almost all piliated. Several hundred individual cells of the *S. typhosa* 643L+ parent were examined and none were found to be piliated, nor were any fragments of pili ever seen on the supporting collodion film (Fig. 2). Over a hundred cells of the original strain, *S. typhosa* 643, were examined and pili were completely absent in this strain also.

Practically all cells of the hybrid X30D, however, were found to be richly piliated as were cells of X30P, while the X30T segregant, which fails to cross-react with *E. coli* showed a complete absence of pili (Fig. 2). Thus, the serological cross-reaction of the hybrid X30D and its segregant X30P with the *E. coli* K-12 Hfr parent, as well as the lack of cross-reaction of the hybrid segregant X30T with the *E. coli* parent, can be explained by the observation that pili are common to the cross-reacting strains.

The pili of these strains were very similar in size, shape, and distribution to each other and to the pili of many other strains. They were of a uniform diameter of about 100 Å, with lengths from less than 0.1 μ to about 3.0 μ, and were distributed uniformly. They were fairly straight and did not exhibit the regular wave-like structure of *flagella*. All of the strains, both parents and hybrids were flagellated.

Serological studies

The results obtained with antisera to the W1895 strain of *E. coli* have been described briefly above and are covered in detail by BARON *et al.*[8]. Further confirmation of the distribution of pilial antigens among the six strains examined, however, was achieved by means of agglutination tests of each strain against antiserum made with purified pili from *E. coli* B–L(E). This rabbit antiserum was prepared using pili which had been mechanically detached from the bacteria and purified by electrophoresis[3]. It agglutinated the piliated phase of *E. coli* B–L(E) to a titer of 1:2560, but did not agglutinate the non-piliated phase. The results of a tube agglutination



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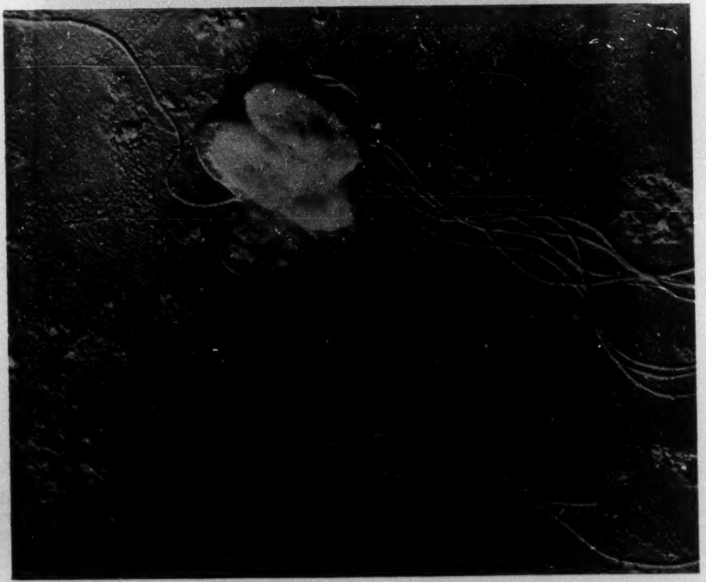

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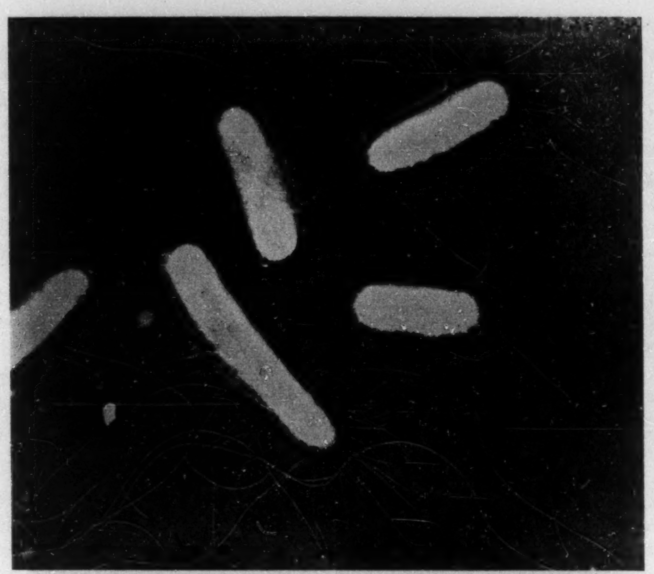

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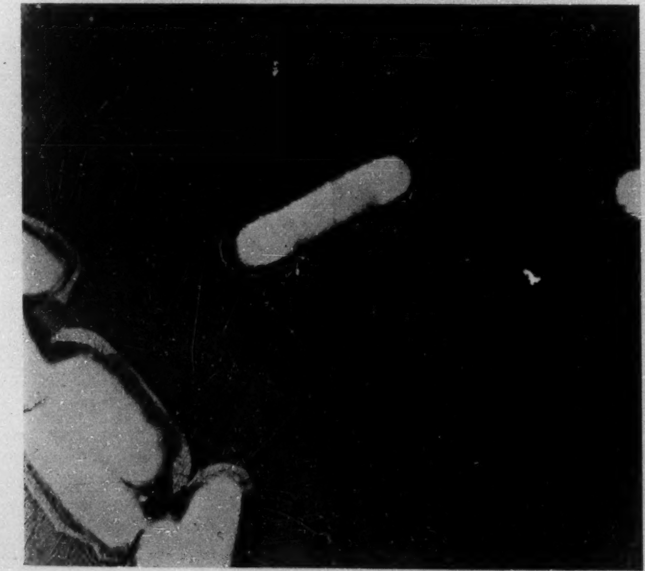

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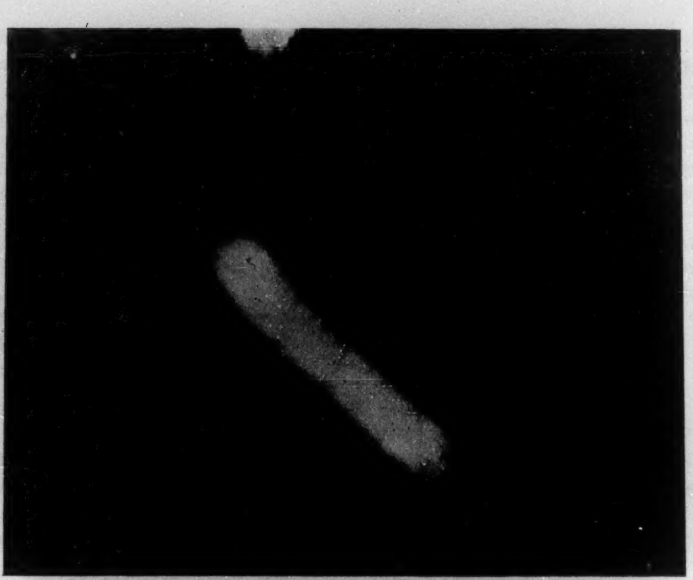

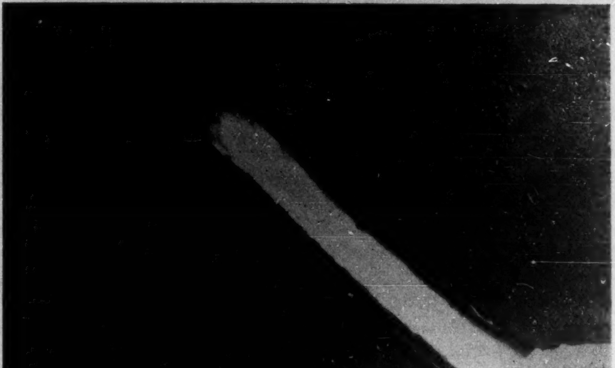

Fig. 2. Electron micrographs. The organisms in Fig. 2b, *S. typhosa* 643L+, were fixed in broth culture and dialyzed against distilled water. The other specimens were washed with distilled water before fixation. a, original *S. typhosa* 643; b, female parent, *S. typhosa* 643L+; c, male parent, *E. coli* K-12, Hfr, W1895; d, hybrid X30D; e, hybrid X30P (segregant from X30D); f, hybrid X30T (segregant from X30D).

test are summarized in Table II, showing that P+ strains, as established in the electron microscope, are agglutinated while P— strains are not agglutinated.

TABLE II

AGGLUTINATION OF PARENT AND HYBRID STRAINS WITH ANTISERUM PREPARED AGAINST PURIFIED PILI FROM *E. coli* B–L(E) P+

Strains	*Antiserum dilutions*				
	4	*16*	*64*	*256*	*1024*
E. coli B–L(E) P+	+	+	+	+	+
E. coli B-L(E) P—	—	—	—	—	—
E. coli K12 Hfr W1895	+	+	+	+	+
Salmonella typhosa 643	—	—	—	—	—
Salmonella typhosa 643L+	—	—	—	—	—
Salmonella X30D (P+)	+	+	+	+	+
Salmonella X30T (P—)	—	—	—	—	—
Salmonella X30P (P+)	+	+	+	+	+

Hemagglutination studies

The phenomenon of agglutination of red blood cells by piliated strains of bacteria[10] provides additional confirmation of the distribution of pili among the parents and hybrids of this cross. Agglutination of chicken red blood cells by cultures of the various strains is indicated in Table III. The *E. coli* parent and the two piliated hybrid forms, X30D and its segregant, X30P, show strong hemagglutination, while the *Salmonella* parents, 643 and 643L+, and the non-piliated hybrid, X30T do not cause hemagglutination even at high ratios of bacterial cells to blood cells. Since hemagglutination has been shown to be due to the presence of the pili themselves, rather than some other material produced by piliated bacteria[3], the results of this test can be taken to indicate clearly the presence or absence of piliation in the various strains.

TABLE III

HEMAGGLUTINABILITY* OF PARENT AND HYBRID STRAINS

Strains	*Dilutions***													*Hemagglutinability (bacterial cells/ blood cells)*
	1	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	*1024*	*2048*	*4096*	
E. coli K12 Hfr W1895 "R"	+	+	+	+	+	+	+	+	±	—	—	—		12
E. coli K12 Hfr W1895 "S"	+	+	+	+	+	+	+	+	±	—	—	—		12
S. typhosa 643	—	—	—	—	—	—	—	—	—	—	—	—		>1540
S. typhosa 643L+		—	—	—	—	—	—	—	—	—	—	—	—	> 770
Salmonella X30D (P+)		+	+	+	+	+	+	+	+	+	+	±	±	1.5
Salmonella X30T (P—)		—	—	—	—	—	—	—	—	—	—	—	—	> 770
Salmonella X30P (P+)			+	+	+	+	+	+	+	—	—	—	—	6.0

* Bacteria per blood cell just needed to give complete agglutination expressed as the ratio of bacterial cells to blood cells.

** Bacterial dilution of 1 corresponds to $2 \cdot 10^{10}$ bacteria/ml.

Biochemical characteristics

Table I also shows the biochemical characteristics of the parent strains and indicates that the hybrids have retained the nutritional requirements typical of the recipient parent strain. Also evident are certain differences between the hybrid X30D, and the segregant forms which arise from it, X30T and X30P. These changes are as follows: X30T is P— and Ara—, while X30P, still P+ and Ara+, differs only in the loss of the Vi antigen. If the loss of pili and the inability to utilize L-arabinose were merely a fortuitous association, linkage of these determinants would not be indicated. That P and Ara are probably closely linked, however, is demonstrated by the fact that more than one hundred separately isolated X30T segregants were both P— and Ara—. Also, a number of Ara+ reversions obtained by plating X30T on EMB arabinose plates were tested. These were all still P— indicating that pili production and arabinose utilization need not always be associated, as would be expected in the case of pleiotropy.

Mutation rate studies

To demonstrate conclusively that the ability of hybrids X30D and X30P to produce *E. coli* cross-reacting pili has been acquired by recombination, it must be shown that the presumably non-piliated *Salmonella* parent could not have mutated to a form with *coli* cross-reacting pili before recombination occurred. Such a mutation is, *a priori*, extremely unlikely, since strains of *Salmonella* do not cross-react with *E. coli*. Even if *Salmonella* 643L+ mutated to a piliated form, the probability of these pili also being *E. coli*-specific is extremely low. GILLIES AND DUGUID[2] have reported that antiserum against the pili of a strain of *Shigella flexneri* strongly agglutinated a series of piliated strains of *Escherichia coli* but did not agglutinate piliated cultures of *Salmonella typhi*, *S. paratyphi* B, *S. enteriditis*, *S. typhimurium*, and *S. thompson*.

A large number of cells of the parent 643L+ culture were examined for the possible presence of pili. Out of 300 individual cells directly examined by electron microscopy, none were piliated. However, this method of examination is very time consuming and other, more efficient methods were employed. Since many strains of bacteria existing as both P+ and P— forms show a parallel colony difference, one can often detect piliated variants among large numbers of non-piliated colonial types[3]. However, out of a total of about 6000 colonies examined by reflected light, only three were clearly different from the typical 643L+ colonies. Two were somewhat smaller with the same surface texture as the original form, and one was about the same size with a rougher surface texture. None of these three colonies were hemagglutinating at a concentration of about 10^{10} cells per ml in saline, nor were the typical 643L+ colonies hemagglutinating. Furthermore, neither the mutant colonial forms nor the normal 643L+ colonial forms showed the rapid colonial breakdown upon subculture as did the hybrid forms. In a third kind of examination, 871 tubes containing 0.5 ml of broth were inoculated with an average of 10 643L+ bacteria each and were grown up to a concentration of about 10^9 organisms per tube. Saline washed chicken red blood cells were added to a concentration of $5 \cdot 10^6$ per tube. No hemagglutination was observed in any of the tubes.

Pooling the above data, out of about 7000 individual cells, colonies, or clones of parent strain 643L+ examined, none showed any evidence of piliation. Thus, the presumably non-piliated parent culture could not have contained more than about 10^{-4} piliated cells per non-piliated cell. However, nearly all the colonies selected on xylose and rhamnose were hybrids with *coli*-specific pili. Since xylose and rhamnose utilization are characters independent from pili production, these hybrids must have acquired their ability to produce pili by transfer of genetic material.

DISCUSSION

The results of the preceding experiments have demonstrated that the ability to produce pili can be transferred from a donor cell to a recipient by means of sexual recombination. In addition, the pili production character is linked to at least one other genetic marker, arabinose utilization. Thus, the production of pili can be under nuclear genetic control, as opposed to an inheritance deriving exclusively from the cytoplasm. It may now be possible to analyze what type of mechanism, whether nuclear, cytoplasmic, or both, has determined the presence or absence of pili in strains of *E. coli* which show a high rate of variation from P+ to P—. Previous studies[3] have shown that other strains can exist in these two alternative forms, and that the rate of change from one form to the other can be high (10^{-4}—10^{-2} per bacterium per division) compared to ordinary mutation rates. In one strain, *E. coli* B-L(E), the P+ to P— rate of spontaneous change is 10^{-3} per bacterium per division at 32° and decreases by a factor of 10 for 10° rise in temperature.

These results seem to suggest a pattern of inheritance of pili possibly more complex than simple mutation and reversion at a single gene locus on the genetic map of this organism. The term "mutation" is used here in the formal sense, and refers to a sudden, random, inheritable change having a constant probability of occurrence per bacterium per generation. Changes of this nature, however, can occur by mechanisms other than that of ordinary spontaneous mutation. Some of these possibilities would be the segregation of diploid heterozygotes after recombination[11], the segregation of heterogenotes after transduction by phage[12], variation in the "local state" of a gene locus as in phase variation in *Salmonella*[13], and ordinary mutation. All of these mechanisms of cellular variation produce clonally stable variants in a stochastic manner. A mathematical analysis of the "mutation" rate and a study of the distribution of "mutants" by the fluctuation test of LURIA AND DELBRUCK[14] would not suffice to distinguish among the above possibilities. Recombinational analysis, however, can provide the necessary tool for this purpose, and investigations along these lines are now in progress.

The frequencies with which P+ recombinants occur when hybrids from a mating between P+ and P— parents are selected for various other traits has not yet been determined with sufficient accuracy to justify assigning the P+ character to a definite locus on the bacterial linkage group. Previous results by BARON *et al.*[8] with strain X30D, however, have dealt with the "diploid" nature of this culture and its resemblance to the heterozygotes reported in *E. coli* by LEDERBERG[11]. The segregant, X30T, a stable haploid form of X30D which is P—, is also Ara—; this is not fortuitous since all other segregants of the X30T variety were found to be invariably Ara— as well as P—. The P+ stable haploid segregant, X30P, derived from the unreduced diploid X30D is an Ara+ strain, which has lost the Vi antigen. During the reduction of this partial diploid (X30D), cross-overs presumably have occurred which, in the first instance (X30T) have resulted in the elimination of the P+ character as well as the Ara+ marker, probably indicative of the close linkage of these two markers. That this may explain the situation entirely can be surmised from previous findings which eliminate the possibility of pleiotropism, since the majority of hybrids selected for Ara+ still remained P—. Thus, recombinants from a mating of *S. typhosa* hybrid 643L+ with *E. coli* Hfr, selected for Ara+ were never found to be P+ unless they possessed as well the identical biochemical characteristics observed in the X30D form or the other more unstable types (Lac+, Ara+, Xyl+, Rha+, Indol+). These were diploid forms which showed antigenic cross-reactivity with *E. coli* and were readily identifiable. In addition, strains of *S. typhimurium* which are P+ have been isolated as a consequence of *in vivo* matings of *E. coli* and *S. typhimurium* in mice[15]. Such P+ forms of *S. typhimurium* were invariably able to produce indol, indicating extensive transfer of genetic material from *E. coli*, since the linkage relationship sequence as studied by BARON *et al.*[9] seems to be Lac–Ara–Inos–Rha–Indol. Indol producing hybrids are obtained following selection for Rha+ hybrids, but have never been detected after Lac+ or Ara+ selections.

Before the production of pili can occur, the results would seem to indicate a necessity for more extensive transfer of genetic material than usually takes place in selection of Ara+ hybrids, although loss of pili can be correlated with a locus linked to arabinose utilization. The reversion of Ara— P— strains (X30T) to Ara+ strains which still remain P— also eliminates the possibility of a pleiotropic effect.

It is possible that a further approach to the genetics of pili can be made by means of transduction studies using the Vi typing phages. Vi phage C_2 has been propagated on hybrid X30D (see ref. 16), and could be used to transduce various markers (Lac, Ara, Xyl, Rha) in an attempt to determine possible linkage relationships of loci controlling pili production. Studies of this nature and further work with the transfer of pili by genetic recombination procedures are in progress.

REFERENCES

[1] C. C. BRINTON, JR., A. BUZZELL AND M. A. LAUFFER, *Biochim. Biophys. Acta*, 15 (1954) 452.
[2] R. R. GILLIES AND J. P. DUGUID, *J. Hyg.*, 56 (1958) 303.
[3] C. C. BRINTON JR., *Nature*, 183 (1959) 782.
[4] E. L. TATUM AND J. LEDERBERG, *J. Bacteriol.*, 53 (1947) 673.
[5] N. D. ZINDER AND J. LEDERBERG, *J. Bacteriol.*, 64 (1952) 679.
[6] L. L. CAVALLI, *Boll. ist. sieroterap, Milan*, 29 (1950) 281.
[7] L. S. BARON, W. F. CAREY AND W. M. SPILMAN, *Proc. VIIth Intern. Congress for Microbiology*, 1958.
[8] L. S. BARON, W. M. SPILMAN AND W. F. CAREY, *Bacteriol. Proc. 59th General Meeting*, 1959.
[9] L. S. BARON, W. F. CAREY AND W. M. SPILMAN, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 976.
[10] J. P. DUGUID, I. W. SMITH, G. DEMPSTER AND P. N. EDMUNDS, *J. Pathol. Bacteriol.*, 70 (1955) 335.
[11] J. LEDERBERG, *Proc. Natl. Acad. Sci. U.S.*, 35 (1949) 178.
[12] M. L. MORSE, E. M. LEDERBERG AND J. LEDERBERG, *Genetics*, 41 (1956) 758.
[13] J. LEDERBERG AND T. IINO, *Genetics*, 41 (1956) 743.
[14] S. E. LURIA AND M. DELBRUCK, *Genetics*, 28 (1943) 491.
[15] H. SCHNEIDER, S. B. FORMAL AND L. S. BARON, *Bacteriol. Proc. 60th General Meeting*, 1960.
[16] L. S. BARON, S. B. FORMAL AND W. SPILMAN, *J. Bacteriol.*, 69 (1955) 177.

ISOLATION OF A PHOSPHOPEPTIDE AS MAGNESIUM COMPLEX FROM A TRYPSIN HYDROLYSATE OF α-CASEIN BY ANION EXCHANGE CHROMATOGRAPHY

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SUMMARY

A phosphopeptide has been isolated from a short-time hydrolysate of α-casein by anion exchange chromatography, using an eluent containing a complex-forming ion (Mg^{2+}). This peptide contains at least 16 % of α-casein phosphorus and is very similar to a peptide previously[1] isolated from this source by zone-electrophoresis. The empirical residue formula is suggested to be: HOOC–Lys_1, Asp_3, Thr_1, Ser_6, Glu_{10}, Pro_1, Gly_1, Ala_2, Val_2, Met_2, $Ileu_4$, Lys_1, Asp–NH_2, $(NH_3)_3$, $(PO_4H_3)_7$. The presence of two lysine residues is discussed in relation to trypsin resistant bonds involving a lysine carboxyl group.

INTRODUCTION

In a previous work[1] from this institute a strongly acidic phosphopeptide was isolated from a tryptic digest of α-casein by zone electrophoresis and its main composition determined*. Further studies of the structure and properties of this peptide require relatively large amount of material. Ion exchange chromatography seemed therefore in this case to imply a more convenient separation method.

Using conventional buffers, however, the separation of the most acidic phosphopeptides of a tryptic α-casein hydrolysate is not entirely satisfactory by this method, reasonably due to the great net negatively charge on these peptides. In preliminary experiments with Dowex 50-X2 and 0.01 *M* HCl as a buffer, these peptides were eluted as a mixture near to the front (*cf.* ref. 2, 3). On Dowex 1-X2 they were strongly bound and could not be recovered with 2 *M* sodium formate (*cf.* ref. 2).

A satisfactory separation was, however, obtained with anion exchange chromatography (Dowex 1-X2), when an eluent containing a complexing metal ion (Mg^{2+}) was used. Magnesium, which has an intermediate tendency to complex formation with O-phosphorylserine[4], was selected, since it seemed that it would provide sufficient blocking of charges, as well as allowing simple removal of bound metal ions. By chromatography of a trypsin digest of α-casein in this way a phosphopeptide was isolated, which contained at least 16 % of the α-casein phosphorus and is apparently the same peptide prepared previously[1]. Its composition has now been confirmed.

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* Recent literature in this field was reviewed in ref. 1.

EXPERIMENTAL AND RESULTS

Trypsin digestion

α-casein (21.8 g, prepared according to Hipp *et al.*[5]; P: 0.99%; homogenous in moving boundary electrophoresis) was treated with Armour's twice recrystallized trypsin (containing 50% $MgSO_4$) for 65 min at 25° and pH 8.0. The procedure and apparatus employed were the same as described previously[1]. From the heat-inactivated digestion solution, a crude barium phosphopeptide fraction (6.1 g) (P: 2.88; N: 9.43%; N/P (atomic ratio): 7.25; 81.5% of total α-casein P) was prepared according to Mellander[6] (excluding the lead treatment).

Column chromatography on Dowex 1-X2 involved three purification steps (Fig. 1).

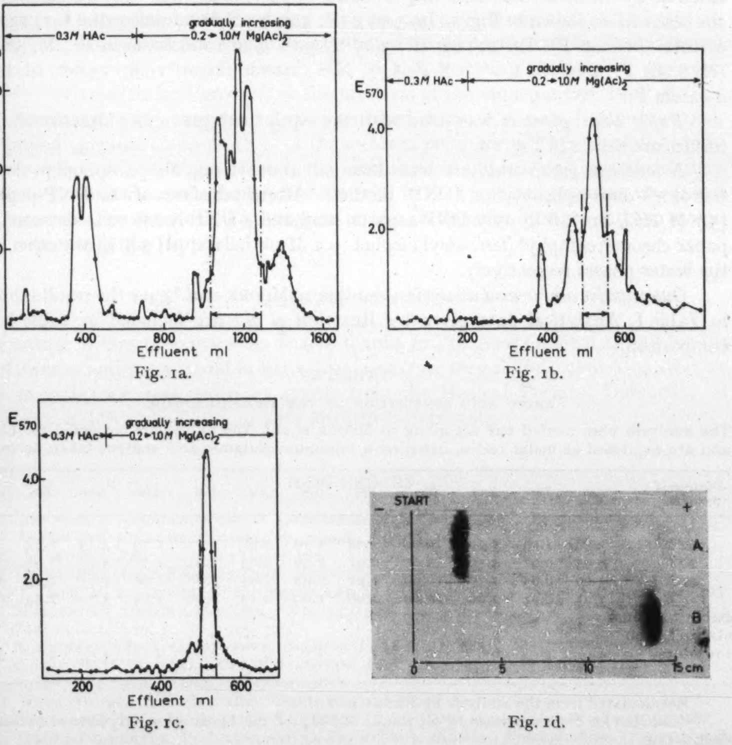

Fig. 1a.

Fig. 1b.

Fig. 1c.

Fig. 1d.

Fig. 1. Chromatography on Dowex 1-X2 of phosphopeptides from a trypsin α-casein hydrolysate, and paper electrophoreses of a purified fraction. ○—○, ninhydrin colour at 570 mμ, given by 0.1 (a) or 0.2 (b, c) ml aliquots of the effluent fractions after alkaline hydrolysis (100°, 2 h) according to Hirs *et al.*[14]. (a) Chromatography of 5.0 g crude barium phosphopeptide on a 2.8 × 40 cm column. (b) Re-chromatography of 300 mg Mg-phosphopeptide, obtained from (a) on a 1.5 × 55 cm column. (c) Re-chromatography of 115 mg Mg-phosphopeptide, obtained from (b) on a 0.9 × 115 cm column. (d) Paper electrophoreses at 23° of about 0.5 mg Mg-phosphopeptide from (c) (ninhydrin colour) in: (A) 0.4 *M* acetic acid (500 V, 5 h) (B) 0.2 *M* formic acid adjusted to pH 2.8 with triethylamine (400 V, 5 h).

The resin, 200–400 mesh, was used in the acetate form. The phosphopeptide was applied in 0.3 M acetic acid and the column was eluted with the same solvent and later with magnesium acetate solution of gradually increasing concentration. The mixing chamber (volume, 1 l), which initially contained 0.2 M magnesium acetate was connected to an upper chamber, filled with M magnesium acetate. Several experiments were carried out in respect of the re-chromatographic runs, and the main shape of the effluent curves was always the same. The peptide fractions were combined as indicated by the vertical lines in Fig. 1 and concentrated to small volume by lyophilisation. Magnesium phosphopeptide was then precipitated by adding 99.5 % ethanol. The precipitate was washed with 99.5 % ethanol and ether and then dried at 45° *in vacuo*. The following amounts of phosphopeptide were obtained from the separations shown in Fig. 1: Ia, 3.99 g (P: 3.24 %; N/P (atomic ratio) 6.1; 73.1 % of total α-casein P); Ib, 125 mg (P: 3.25 %; 30.6 % of total α-casein P); Ic, 58 mg (N: 8.66; P: 3.33; S: 1.0 %; N/P: 5.75, N/S (atomic ratio): 19.8; 15.8 % of total α-casein P).

Paper electrophoresis was done with the equipment previously described[3]. The results are shown in Fig. 1d.

N-terminal group analysis was done on about 2 mg Mg-phosphopeptide by SANGER's dinitrophenylation (DNP) method[7]. After hydrolysis of the DNP-peptide (5.7 M HCl, 110°, 8 h) only DNP-aspartic acid and ε-DNP-lysine were detected by paper chromatography[8] (*tert.*-amyl alcohol–0.1 M phthalate, pH 5.5) of the ether and the water phases respectively.

Quantitative amino acid analysis according to MOORE *et al.*[9] gave the results shown in Table I. Analytical data given by BENNICH *et al.*[1] are included in Table I for comparison.

TABLE I

AMINO ACID COMPOSITION OF THE PHOSPHOPEPTIDE

The analyses were carried out according to MOORE *et al.*[9]. The values are corrected for blank and are expressed as molar ratios, based on a minimum glutamic acid content taken as 10.00.

Duration of hydrolysis	*Asp*	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Val*	*Met*	*Ileu*	*Lys*	NH_3
22 h*	3.78	1.03	5.22	10.00	1.24	1.19	2.06	1.75	1.73	3.23	2.74	
22 h	4.05	0.91	4.56	10.00	0.97	1.06	2.63	1.84	1.72	3.47	1.95	3.92**
72 h	3.96	0,85	4.29	10.00	1.21	0.92	2.03	2.00	1.98	3.67	2.03	3.77**
144 h	3.75	0.94	2.90	10.00	1.06	1.09	2,13	2.06	1.64	3.70	2.34	3.14**
Probable minimum number of amino acid residues	4	1	6	10	1	1	2	2	2	4	2	3***

* Recalculated from the analysis by BENNICH *et al.*[1].

** Corrected for contamination by 0.4 μmole of NH_3 (*cf.* ref. 14) and for NH_3 formed by serine destruction.

*** Tentative value of amide groups.

DISCUSSION

Characterisation and analyses indicate that a homogenous phosphopeptide can easily be prepared from a tryptic α-casein hydrolysate by anion exchange chromatography, using a buffer with a sufficient concentration of magnesium ions. The presence of these complexing ions seems to be necessary for the purification of this phosphopeptide, though phosphopeptides with less acidic properties are well separated in more conventional buffer systems (see *e.g.* STRID[10]).

The peptide isolated, probably has a minimum amino acid composition of Asp_4, Thr, Ser_6, Glu_{10}, Pro, Gly, Ala_2, Val_2, Met_2, Ileu_4, Lys_2, $(\text{NH}_3)_3$, $(\text{PO}_4\text{H}_3)_7$*, with aspartic acid in the N-terminal position. It is apparently the same peptide as that previously prepared[1] by zone electrophoresis. It was then reported to contain at least one and possibly two lysine residues. The present investigation establishes a minimum of two lysine residues.

In view of trypsin specificity, one of these lysine residues should occupy the C-terminal position. The occurrence of a second lysine residue may indicate the presence of a peptide bond involving a lysine carboxyl group that is resistant to hydrolysis. This resistance could be attributed to protection of the bond by an adjacent negatively charged group[11], *i.e.* in this case a phosphoryl-[12], glutamic acid-[13,14] or aspartic acid group[15], or the presence of the sequence Lys·Pro[14,16–18].

According to the analytical figures obtained during the course of purification, the present peptide contains 16 % of the α-casein phosphorus. This percentage may be somewhat too low, since losses in the precipitation and washing procedures may reach 10 %, and since only the peptide material in the combined fractions was taken into account. Further work is in progress on the phosphorus linkages and the amino acid sequence of the peptide.

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REFERENCES

[1] H. BENNICH, B. JOHANSSON AND R. ÖSTERBERG, *Acta Chem. Scand.*, 13 (1959) 1171.
[2] G. ÅGREN AND J. GLOMSET, *Acta Chem. Scand.*, 7 (1953) 1071.
[3] R. ÖSTERBERG, *Arkiv Kemi*, 13 (1959) 409.
[4] R. ÖSTERBERG, *Arkiv Kemi*, 13 (1959) 393.
[5] N. J. HIPP, M. L. GROVES, J. H. CUSTER AND T. L. MCMEEKIN, *J. Dairy Sci.*, 35 (1952) 272.
[6] O. MELLANDER, *Upsala Läkarfören. Förh.*, 52 (1947) 107.
[7] F. SANGER, *Biochem. J.*, 39 (1945) 507.
[8] S. BLACKBURN AND A. G. LOWTHER, *Biochem. J.*, 48 (1951) 126.
[9] S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
[10] L. STRID, *Acta Chem. Scand.*, 13 (1959) 1787.
[11] H. NEURATH AND G. W. SCHWERT, *Chem. Revs.*, 46 (1950) 69.
[12] D. THEODOROPOULOS, H. BENNICH, G. FÖLSCH AND O. MELLANDER, *Nature*, 184 (1959) 187.
[13] J. JOLLÈS-THAUREAUX, P. JOLLÈS AND C. FROMAGEOT, *Biochim. Biophys. Acta*, 27 (1958) 298.
[14] C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 219 (1956) 623.
[15] I. I. GESCHWIND, C. H. LI AND L. BARNAFI, *J. Am. Chem. Soc.*, 79 (1957) 620.
[16] R. G. SHEPHERD, S. D. WILLSON, K. S. HOWARD, P. H. BELL, D. S. DAVIES, S. B. DAVIS, E. A. EIGNER AND N. E. SHAKESPEARE, *J. Am. Chem. Soc.*, 78 (1956) 5067.
[17] J. I. HARRIS, *Biochem. J.*, 71 (1959) 451.
[18] D. T. GISH, *Biochim. Biophys. Acta*, 35 (1959) 557.

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* This composition gives N/P and N/S atomic ratios of 5.72 and 20.0. The analytical data gave 5.75 and 19.8.

PURIFICATION AND SPECIFICITY OF THE C-S-LYASE OF *ALBIZZIA LOPHANTA*

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SUMMARY

Purification of the L-cysteine C-S-lyase of *Albizzia lophanta* seed endosperm has resulted in a 200-fold increase in specific activity as compared with the original extract. The enzyme is stable above pH 6.0 and below 60°, is activated by pyridoxal phosphate, and acts optimally on S-ethyl-L-cysteine at pH 8.0.

A survey of the specificity pattern of the enzyme with 40 potential substrates demonstrate that whereas the L-cysteine moiety, unaltered except for addition of oxygen to the sulfur atom and substitution of the hydrogen of the thiol group, is an absolute requirement for susceptibility to enzyme action, all or almost all S-substituted L-cysteine derivatives tested may serve as substrates, although there is considerable variation in the rate of hydrolysis to free thiol, pyruvate, and ammonia. In general it appears that those S-substituents whose structure suggest interference (either by hydrogen bonding or by steric hindrance) with the formation of a hydrogen bond (or chelate) between the hydroxyl at the 3-position of pyridoxal phosphate and the cysteine-derived group of the Schiff's base are poor substrates.

The utility of C-S lyases in the preparation, elucidation of structure and their possible role in the metabolism of sulfur compounds of biochemical interest are discussed.

INTRODUCTION

The occurrence of enzymes in higher plants capable of hydrolyzing S-substituted derivatives of L-cysteine has been demonstrated in bulbs of the genus *Allium*[1,2] and in the seed endosperm of the ornamental shrub *Albizzia lophanta*[3,4]. The former enzyme (alliinase) is associated in the fleshy tissue of the bulb with relatively large amounts of alliin (and other S-alkenyl and S-alkyl substituted cysteine sulfoxide derivatives) which interact when the tissue is damaged to yield pyruvate, ammonia and presumably unstable alkenyl or alkyl sulfenic acids which are further converted into the corresponding thiolsulfinates (allicin a.o.). The latter enzyme, upon the addition of water, interacts with djenkolic acid present in the endosperm to yield pyruvate, ammonia and highly odiferous substances, presumably methane dithiol and its decomposition products[3].

Although pyridoxal phosphate appears to be the coenzyme for both enzymes[3,5], alliinase hydrolyses only S-substituted derivatives of L-cysteine sulfoxides whereas the C-S-lyase of *Albizzia* appears to exhibit a much broader specificity. Recently the present authors found that this enzyme can convert S-(2,4-dinitrophenyl)-L-cysteine to pyruvate, ammonia and 2,4-dinitrothiophenol[4]. The spectral difference between the substrate and the product in this reaction has served as the basis for the development of a continuous spectrophotometric assay of the enzyme. This assay has been utilized as a guide in its partial purification, procedures for which are presented in the present paper which also reports on some of the properties and on the specificity of action of this enzyme. The results suggest that the variation in reactivity of a large number of S-substituted L-cysteine derivatives can be interpreted in terms of the ability of the side groups of the S-substituents to interfere with formation of a stabilizing hydrogen bond (or chelate) between the amino group and the pyridine hydroxyl of the Schiff's base formed from the interaction of pyridoxal phosphate and the substrate.

MATERIAL AND METHODS

Enzyme source

Seeds of *Albizzia lophanta* were obtained locally from a commerical seed supply firm under the name of *Acacia lophanta*.

Enzyme activity

Details of the method for assaying enzyme activity are given in a recent publication[4]. The assay is based upon the change in absorbancy at 400 mμ due to the enzymic conversion of S-(2,4-dinitrophenyl)-L-cysteine to 2,4-dinitrothiophenol, (after correction for non-enzymic transarylation to N-(2,4-dinitrophenyl)-L-cysteine) at pH 6.5. One unit of activity is defined as that amount of enzyme in 3 ml of reaction mixture in a 1-cm cuvette which will cause the reaction to proceed at an initial rate of 0.054 O.D. units/min corresponding to conversion of 10^{-5} mmoles of substrate.

Enzyme activity was also estimated from the amount of pyruvate formed determined according to the method of MATSUO AND GREENBERG[6] with the following modifications. An aliquot of enzyme digest not greater than 0.1 ml was added directly to the 2,4-dinitrophenylhydrazine reagent and the final color was measured with an EEL colorimeter (green filter). One unit of enzyme will release 0.186 μmoles of pyruvate/min at 37° in 1 ml of enzyme reaction mixture containing an excess (0.05 M) of L-djenkolic acid, 0.02 μmoles pyridoxal phosphate and 0.04 μmoles of borate buffer at pH 8.0.

Potential substrates

A collection of amino acids was obtained from (a) commercial sources, (b) through synthesis and through the generous gifts of the following individuals; (c) G. ALDERTON, (d) J. A. V. ARNSTEIN, (e) R. GMELIN, (f) D. M. GREENBERG, (g) J. B. NEILANDS, (h) H. TUPPY, (i) W. C. J. ROSS, (j) R. G. WESTALL and (k) R. M. ZACHARIUS. The letters in parentheses are used in a subsequent table to identify the source of each compound.

The following substrates were prepared in this laboratory. S-(2,4-dinitrophenyl)-L-cysteine and 2,4-dinitrothiophenol were synthesized according to procedures previously described[4,7]. The heretofore unknown S-benzyl-N-benzoyl-L-cysteine was prepared by treating 3,1 g of S-benzyl-L-cysteine hydrochloride with benzoyl chloride in alkali at 0° and was isolated by acidification and filtration. The precipitate was washed with water and dried *in vacuo* to give 3.0 g of crude product which was purified by recrystallization, first from aqueous ethanol and then from a mixture of ethyl acetate and petroleum ether. An analytical specimen had m.p. 117° (uncorr.) and the following rotations $[\alpha]_D^{24}$ —39.9° (0.5 *N* NaOH), $[\alpha]_D^{24}$ —79.1° (EtOH). (Calc. for $C_{17}H_{17}O_3NS$: C 64.73; H 5.43; N 4.44. Found: C 64.99; H 5.43; N 4.03.)

The diastereoisomeric pair of S-benzyl-DL-thiothreonines, A and B, were prepared from the corresponding N-benzoyl-S-benzyl-DL-thiothreonines. 5 mg of each of these compounds was hydrolyzed in 5 ml of 6 *N* HCl in sealed glass tubes for 2 h at 110° and cooled. The hydrolysates were then passed through a small column containing Dowex-50 which was then washed with water and the amino acids were eluted with 2*N* ammonia. The eluates, after extraction with ether, were evaporated to dryness to yield 2 to 3 mg each of products, both of which exhibited an R_F of 0.73 on paper chromatograms (Whatmann No. 1 filter paper; solvent: *n*-butanol–acetic acid–water (120:30:50); spots detected with ninhydrin reagent). These thiothreonine derivatives were found to be completely refractory to the action of carboxypeptidase. S-ethyl-L-cysteine sulfone was prepared by treatment of 50 mg of S-ethyl-L-cysteine for 24 h at room temperature with 10 ml of 1 % hydrogen peroxide in 99 % formic acid containing 0.001 *M* molybdate. The resulting sulfone was precipitated and washed with ether and dried. A sample, repeatedly recrystallized from 80 % ethanol, had m.p. 180° (uncorr., decomp.). According to paper chromatography in *n*-butanol–acetic acid–water (120:30:50) a slight contamination with the corresponding sulfoxide proved very difficult to remove. The employed specimen, however, was estimated to be more than 98 % pure.

Cysteine derivatives of isobutyric acid were prepared by coupling free cysteine with β,β'-diiodoisobutyric acid (prepared according to the method of CORSE AND JANSEN[8]) in aqueous alkali. This procedure was not entirely satisfactory and better results attended attempts to condense free L-cysteine methyl ester, liberated *in situ* from its hydrochloride by CH_3ONa, and the methyl ester of β,β'-diiodoisobutyric acid in methanol solution containing an additional molecular equivalent of sodium methoxide. The latter ester was used as the oily reaction product formed upon treatment of the diiodoacid with excess of diazomethane in ethereal solution. The coupling proceeded at room temperature and the crude reaction product was saponified with aqueous NaOH for a few hours at room temperature, then neutralized by addition of HI. The anhydrous residue was then repeatedly extracted with absolute ethanol in order to remove NaI and other impurities. The resulting material did not possess any well-defined melting point. It could be purified by repeated precipitations from aqueous solutions at pH 7 by addition of ethanol. The resulting preparation was devoid of free cysteine (nitroprusside negative before and after cyanide) and served as a substrate for *Albizzia* C-S lyase, yielding free thiol, pyruvate and ammonia. However, paper chromatography in *n*-butanol–acetic acid–water (120:30:50) revealed the presence of two ninhydrin positive spots of which the fastest running was undoubtedly the desired condensation product, 3,3′-bis-(2-carboxy-2-aminoethylthio)-isobutyric acid. Various observations suggested that the tenaciously adhering contamination was the analogous 3-(2-carboxy-2-aminoethylthio)-3-hydroxyisobutyric acid formed by competitive hydrolysis of the diiodoacid.

RESULTS

Enzyme purification

200 g of *Albizzia lophanta* seeds were coarsely ground in a coffee mill and then shaken with trichloroethylene. Seed coat settled to the bottom of the container and endosperm, which floated to the top, was removed and dried overnight in the air to remove solvent. The endosperm was ground to give 90 to 95 g of fine meal which was then extracted with 360 ml of water for 1 h at room temperature under a hood. The suspension was squeezed through cheese cloth to yield 290 ml of Fraction A. The pH of fraction A was adjusted to 5.5 (from pH 7.3) with 6 ml of 1 *N* HCl, and the suspension was centrifuged at 2,100 × *g* for 30 min to yield 210 ml of supernate (Fraction B). Fraction B was neutralized with 1 *N* NaOH and heated to $65 \pm 3°$ for 10 min, cooled and then centrifuged at 2,100 × *g* to yield 200 ml of supernate (Fraction C). To Fraction C was added 60 g of ammonium sulfate (0.42 saturated), the suspension was centrifuged at 2,100 × *g* for 30 min and the resulting precipitate was taken up in water to give 25 ml of Fraction D. To Fraction D was added saturated ammonium sulfate to 0.42 saturation at pH 6.54. The resulting precipitate was centrifuged and dissolved in water to give 15 ml of Fraction E which was then dialyzed for 48 h at 5° against distilled water. The precipitate, which started to form after 16 h of dialysis, was centrifuged off at 4,000 × *g* and lyophilized to yield 150 mg of Fraction F. Table I shows the total and specific activity of each fraction of the purification procedure. A 17 % yield of enzyme representing a 70-fold increase in specific activity was obtained.

TABLE I

PURIFICATION OF *Albizzia* L-CYSTEINE C-S-LYASE

Fraction	*Description*	*Volume ml*	*Total units*	*Specific activity units/mg protein*
A	Water extract	290	208,000	3
B	Supernate, pH 5.5	210	70,100	12
C	Supernate, heating	200	66,300	19
D	Ppt, 1st $(NH_4)_2SO_4$	25	47,200	79
E	Ppt, 2nd $(NH_4)_2SO_4$	15	43,900	145
F	Dialysis, lyophilize	—	35,000	210
G	Alumina treatment of Fraction D	20	5,000	680

Further purification could be achieved by adsorption on and elution from alumina. To 25 ml of an enzyme preparation corresponding to Fraction D (Table I) was added 1.6 ml of alumina for chromatography (Sigma). The precipitate from the ensuing centrifugation was washed with 10 ml of water and then eluted with three successive 10 ml portions of 5.0, 9.3 and 13.8 % solutions of ammonium sulfate solutions, respectively. The last two fractions contained about 10 % of the starting activity (Fraction G, Table I) and had specific activities of 643 and 700 units/mg of protein nitrogen, respectively. The turnover number of the most active fraction has been calculated as 13,000 molecules of pyruvate produced from L-djenkolic acid per minute per molecule of enzyme of molecular weight = 100,000 at 37° and pH 8,1 in the presence of $2 \cdot 10^{-5}$ *M* pyridoxal phosphate, a value which compares favorably with the turnover number of 2,340 obtained by Matsuo and Greenberg[6] for the action of liver cystathionase acting on DL-cystathionine.

Properties of crude and purified preparations

Enzyme activity could be detected only in the endosperm. Extracts of the seed coat did not affect the activity of the endosperm extracts. These seed coat extracts solidified to a gel on standing overnight, thus indicating the presence of a strong pectin-pectinesterase system. The density of the seed coat was much greater than that of the endosperm, thus allowing their separation by flotation in inert solvents of relatively high density with little loss of activity. However, the trichloroethylene extractible material exhibited appreciable activity after removal of the solvent, amounting to about 1 % of the total activity present in a water extract of the endosperm.

Centrifugation of the crude extract, after squeezing through cheese cloth, resulted in a residue consisting of a layer of starch granules beneath a loosely packed yellow sediment and a milky yellow supernate. The material responsible for the opalescence could not be sedimented at 18,000 × *g*, readily passed through Celite pads and prevented heat and salt precipitation of the protein. After adjustment of the pH from 7.3 to 5.5, this colloidal material could be almost quantitatively removed by centrifugation at moderate speeds.

The enzyme in the extract was quite unstable below pH 5, *i.e.*, about 50 % of the activity was lost after 40 min at pH 4.0 and 5 %. The loss of activity upon heating the extract at 60°, 70° and at 80° amounted to 5 %, 12 % and 25 %, respectively.

The final preparation (corresponding to Fraction E, Table I)was quite stable. Thus a 0.3 % solution of the purified enzyme in 0.05 *M* phosphate at pH 6.5 retained 80 % of its original activity after 5 months at 5°. The above values on stability were found using the spectrophotometric assay method with S-(2,4-dinitrophenyl)-L-cysteine as substrate[4].

The effect of pH on the activity of the enzyme with S-ethyl-L-cysteine as substrate is shown in Fig. 1. The enzyme exhibited maximal activity between pH 7.8 and 8.2 and was completely inactive below pH 5.0.

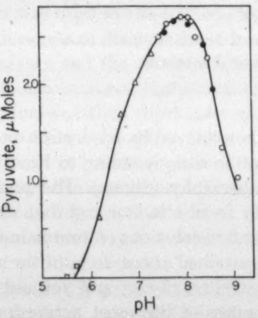

Fig. 1. Effect of pH on activity of *Albizzia* C-S lyase. 1 ml of reaction mixture contained 40 μmoles S-ethyl-L-cysteine, 40 μmoles buffer, 0.05 μmole pyridoxal phosphate and 4.5 units of enzyme. Reaction run for 10 min at 37°. The following buffers were used: ○, borate; ●, tris; △, phosphate; □, acetate.

Table II shows the effects of pyridoxal phosphate, mercaptoethanol and ethylenediamine tetra-acetate (EDTA) on the activity of the enzyme with S-ethyl-L-cysteine as substrate. Of these three substances only pyridoxal phosphate caused an increase in the rate of hydrolysis of the substrate. In the absence of pyridoxal phosphate neither mercaptoethanol nor EDTA influenced the activity. In the presence of pyridoxal phosphate there does appear to be a significant inhibition by these substances.

TABLE II

EFFECT OF MERCAPTOETHANOL AND EDTA ON *Albizzia* C-S-LYASE ACTIVITY

1 ml reaction mixture contained 40 μmoles of S-ethyl-L-cysteine, and 4.5 units of enzyme at pH 8.0 (borate buffer, 0.04 *M*) in the absence of EDTA and pH 7.8 in the presence of EDTA.

Substance added	*µmoles of pyruvate liberated in 10 min at 37°*	
	No pyridoxal phosphate	*Plus pyridoxal phosphate ($5 \cdot 10^{-5}$ M)*
None	2.10	2.82
Mercaptoethanol 0.007 *M*	2.02	2.40
EDTA 0.005 *M*	2.18	2.20
Mercaptoethanol + EDTA	1.60	1.95

TABLE III

EFFECT OF PYRIDOXAL PHOSPHATE ON C-S-LYASE ASSAY SYSTEM

Enzyme units	*Pyridoxal phosphate $M \times 10^{-5}$*	*Increase in absorbancy after**		
		5 min	*9 min*	*30 min*
1.00	0	0.280	0.435	1.03
1.00	1.8	0.315	0.483	—
1.00	18	0.340	0.560	—
0.82	18	0.282	0.430	1.02
1.00**	1.8	0.310	—	—
1.00***	1.8	0.309	—	—

* Reaction mixture which contains S-(2,4-dinitrophenyl)-L-cysteine as substrate, and procedures are those described for the enzyme assay[5].

** Pyridoxal phosphate incubated with substrate 10 min prior to addition of enzyme.

*** Pyridoxal phosphate incubated with enzyme 10 min prior to addition of substrate.

Table III shows the effect of pyridoxal phosphate on the enzyme activity with S-(2,4-dinitrophenyl)-L-cysteine as substrate. The increase percentage in initial velocity was about that observed with S-ethyl-L-cysteine as substrate. When the amount of enzyme in the presence of pyridoxal phosphate was decreased to give an initial velocity equal to that in its absence, no difference in the subsequent course of hydrolysis was observed. Prior incubation of pyridoxal phosphate with either substrate or enzyme did not alter its activating effect.

Specificity

The specificity pattern of the C-S-lyase is shown in Table IV. It appears that the L-cysteine moiety, unaltered except for substitution of the hydrogen of the thiol group and addition of oxygen to the sulfur atom, is an absolute requirement for susceptibility to enzyme action. Thus the following changes resulted in compounds which

TABLE IV

SPECIFICITY OF *Albizzia* C-S-LYASE

Reaction mixture at pH 8.1 (0.04 *N* borate) and 37° contained per ml: 10 μmoles of substrate. 0.02 μmoles of pyridoxal phosphate, 14 units (70 μg) enzyme (fraction E).

Substrate	*Pyruvate μmoles/10 min*	*Substrate*	*Pyruvate μmoles/10 min*
D-cy* (a)**	0.00	Cy-isobutyric acid (b)	2.74
L-cy (a)	1.10	S-(2,4-dinitrophenyl)-L-cy (b)***	0.19
S-methyl-L-cy (e)	4.76	Porphyrin *c* (synth.) (g) §	0.01
S-methyl-L-cy sulfoxide (e)	2.44	L-homocysteine (a)	0.00
S-ethyl-L-cy (e)	5.44	L-methionine (a)	0.00
S-ethyl-L-cy sulfone (b)	0.94	L-penicillamine (d)	0.00
S-propyl-L-cy (e)	6.00	S-benzylthiothreonines A and B (d) §§	0.00
S-benzyl-L-cy (e)	6.90	α-methyl-DL-cy (h)	0.00
Felinine (i)	0.60	N-benzoyl-S-benzyl-L-cy (b)	0.00
S-β-chloroethyl-L-cy (h)	3.92	S-benzyl-α-methyl-DL-cy (h)	0.00
S-β-bromoethyl-L-cy (b)	2.91	Glutathione (a)	0.00
S-β-carboxymethyl-L-cy (b)	0.40	S-methylglutathione (k)	0.00
S-β-carboxyethyl-L-cy (e)	0.26	Serine (a)	0.00
L-lanthionine sulfoxide (e)	1.35	Homoserine (f)	0.00
L-lanthionine (e)	0.75	Threonine (a)	0.00
β-methyl lanthionine (ex subtilin) (c)	0.26		
β,β-dimethyl lanthionine (d)	0.09		
DL-allocystathionine	0.25		
L-djenkolic acid (a)	6.25		
Methyl djenkolic acid (e)	3.20		
Isopropyldjenkolic acid (e)	0.81		
Isobutyldjenkolic acid (e)	0.19		

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* Cy, abbreviation for cysteine.

** Letters in parentheses refer to source of compound as mentioned in MATERIALS AND METHODS.

*** Reaction mixture at 25° contained 0.2 μmole/ml of substrate at pH 6.5.

§ 1 ml digest at 37° and pH 8.1 contained 0.5 μmole substrate, 48 units of enzyme and 0.02 μmoles of pyridoxal phosphate.

§§ N-benzoyl-S-benzyl-L-thiothreonines and S-benzyl-L-thiothreonines used.

are not substrates: introduction of CH_2 into the chain (homocysteine); substitution of the α-hydrogen (α-methyl-DL-cysteine); substitution of one or more β-hydrogens (S-benzyl thiothreonine, penicillamine); substitution on the nitrogen (S-benzyl-N-benzoyl-L-cysteine, glutathione, S-methyl glutathione); replacement of the sulfur with oxygen (serine).

Alkylation of the sulfur atom increased the rate of hydrolysis (propyl > ethyl > methyl > hydrogen). Carboxylation of the terminal end of the S-alkyl group resulted in a drastic reduction of susceptibility to enzyme action, *i.e.*, compare S-ethyl-L-cysteine with β-carboxyethyl-L-cysteine and lanthionine. Halogenation resulted in only a moderate reduction in susceptibility. On the other hand, substitution of S-alkyl-α-hydrogen by an S-cysteine group resulted in an increase in the rate of hydrolysis as exemplified by L-djenkolic acid. It will be noted that another member of this category, "cysteine-isobutyric acid", was also cleaved rapidly.

In general it appears that substitution at the carbon atom, α to the sulfur in the S-substituent, decreased the susceptibility to hydrolysis. Thus felinine, S-1,1-dimethyl-3-hydroxypropyl-L-cysteine[9] was a much poorer substrate than S-propyl-L-cysteine; methyllanthionine and dimethyllanthionine (unsymm.) are poorer substrates than lanthionine; the substituted djenkolic acids are less susceptible to hydrolysis than was djenkolic acid; and synthetic porphyrin *c* (see ref. 10), apparently identical with that from cytochrome (see ref. 11) was hydrolyzed at a negligible rate. Terminal arylation of the alkyl chain enhanced the hydrolysis rate (S-benzyl-L-cysteine) and direct arylation of the sulfur atom does not prevent hydrolysis (S-(2,4-dinitrophenyl)-L-cysteine).

DISCUSSION

While the presence of the L-cysteine moiety in the substrate is an absolute requirement for action of *Albizzia* C-S-lyase, it appears that in general all or most S-substituted L-cysteines may serve as substrates although there is a considerable variation in the rate of hydrolysis, depending upon the structure of the S-substituent. Although many factors probably enter into the determination of susceptibility to hydrolysis as a function of structure of the S-substituent, inspection of molecular models of the Schiff's base formed from interaction of the L-cysteine derivatives with pyridoxal phosphate suggest a rational basis for this variation, *viz.*, the substitution of carboxyl groups in the terminal end of the S-alkyl-substituents and the introduction of branching groups in the carbon atom α to the sulfur, interfere with hydrogen bond (or chelate) formation between the hydroxyl at the 3-position of the pyridoxal and the imino nitrogen of the Schiff's base derived from the α-amino group of the amino acid. According to SNELL[12] this stabilizes the aldoxime structure, and otherwise facilitates the subsequent removal of electrons to the pyridine nitrogen, thereby labilizing the α,β-bond in the amino acid. The terminal carboxyl is present in a spatial relation to the imino nitrogen in such a manner that it is capable of forming a hydrogen bond with the latter, thus competing with the pyridine-substituted hydroxyl, whereas the position of the α-substituents suggests steric hindrance to the formation of such a bond. A model of lanthionine sulfoxide, which is a much better substrate than lanthionine, reveals that the bond angle of the sulfur is altered to a degree such that the above-mentioned hydrogen bond formation is much less likely than in lanthionine. A similar relation holds for the carboxyl groups of djenkolic acid.

In connection with the reactivity of the L-cysteine sulfoxides, of which there exist two stereoisomers, due to the asymmetry of the SO group, STOLL AND SEEBECK[1] found that alliine ((+)S-allyl-L-cysteine sulfoxide) was hydrolyzed more rapidly than (—)-S-allyl-L-cysteine sulfoxide by garlic alliinase. This finding is an exception to the general rule that either only one of two stereoisomeric forms of a substance serves as substrate for an enzyme or that the two isomers serve equally well as substrates if the optically active center is not involved in the enzyme action. Molecular models of the two isomers of the Schiff's base of these diastereoisomers show that the S-oxygen of one form interferes to a greater extent with the above-mentioned chelate or hydrogen bond formation than does the alternate form.

The extremely high rate of hydrolysis of S-benzyl-L-cysteine suggests that the benzene ring enhances the electron-attracting function of the heterocyclic nitrogen, thus facilitating electron displacement from the α-carbon atom.

The specificity pattern exhibited by liver cystathionase[13] differs strikingly from that of *Albizzia* C-S-lyase in that (a) the former forms cysteine and α-ketobutyric acid from L-cystathionine (b) homoserine and homocysteine are substrates for cystathionase but not for C-S-lyase; (c) the S-alkyl-L-cysteines are much poorer substrates for cystathionase than are L-djenkolic acid and cystathionine. These differences may reflect differences in the mode of elimination. SNELL[12] considers cystathionase action as an example of γ-elimination whereas the *Albizzia* C-S-lyase action can best be explained as proceeding via an α,β-elimination mechanism. The enzyme also differs from cystathionase in its pH optimum and in the effect of mercaptoethanol on the activity[14].

The properties of *Albizzia* C-S-lyase indicate that it may find some use in the preparation and elucidation of the structure of sulfur compounds of biochemical interest. Thus the refactoriness of synthetic porphyrin *c* indicates substitution at the carbon atom of the porphyrin moiety α to the sulfur atom and thus constitutes corroborative evidence for the identity of this compound with that obtained from cytochrome *c*. The thiol resulting from the action of this enzyme on felinine (1,1-dimethyl-3-hydroxypropanethiol) may be of interest in mevalonic acid metabolism. Action of this enzyme on the β-methyl lanthionines from subtilin[15] and on that from yeast[16] should yield thiothreonines A and B[17], the absolute configurations of which have recently been established by ARNSTEIN[18]. Thus the absolute configuration of the substituted β-carbon of these two lanthionines can likewise be established. Correspondingly, enzymic hydrolysis of *meso*-lanthionine might be employed for the preparation of D-cysteine. The rapid hydrolysis of the dicysteine derivative of β,β-dithiolisobutyric acid to the free dithiol (which occurs in asparagus[19]) suggests a possible pathway of biosynthesis of this and other naturally occurring dithiols such as lipoic acid. MELVILLE *et al.*[20], showed that only the sulfur of cysteine is incorporated into ergothioneine, a finding which is consonant with the possibility of C-S-lyase action in the biosynthesis of this compound.

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REFERENCES

[1] A. STOLL AND E. SEEBECK, *Advances in Enzymology*, Vol. XI, Interscience Publishers, Inc., New York, 1951, p. 377.

[2] A. I. VIRTANEN, *Schweiz. Z. allgem. Pathologie u. Bakteriol.*, 21 (1958) 970.

[3] R. GMELIN, G. HASENMAIER AND G. STRAUSS, *Z. Naturforsch.*, 12 (1957) 687.

[4] S. E. HANSEN, A. KJÆR AND S. SCHWIMMER, *Compt. rend. trav. Lab. Carlsberg Ser. chim.*, 31 (1959) 193.

[5] E. V. GORYACHENKOVA, *Doklady Acad. Nauk SSSR*, 87 (1952) 475.

[6] Y. MATSUO AND D. M. GREENBERG, *J. Biol. Chem.*, 230 (1959) 545.

[7] H. ZAHN AND K. TRAUMANN, *Z. Naturforsch.*, 9b (1954) 578.

[8] J. CORSE AND E. F. JANSEN, *J. Am. Chem. Soc.*, 77 (1955) 6632.

[9] R. G. WESTALL, *Biochem. J.*, 55 (1953) 244.

[10] J. B. NEILANDS AND H. TUPPY, *Biochim. Biophys. Acta*, 38 (1960) 351.

[11] K. G. PAUL, *Acta Chem. Scand.*, 5 (1951) 389.

[12] E. E. SNELL, *Vitamins and Hormones*, Vol. XVI, Academic Press, Inc., New York, 1958, p. 78.

[13] Y. MATSUO AND D. M. GREENBERG, *J. Biol. Chem.*, 234 (1959) 516.

[14] Y. MATSUO AND D. M. GREENBERG, *J. Biol. Chem.*, 234 (1959) 507.

[15] G. ALDERTON AND H. FEVOLD, *J. Am. Chem. Soc.*, 73 (1951) 463.

[16] P. F. DOWNEY AND S. BLACK, *J. Biol. Chem.*, 228 (1951) 171.

[17] H. E. CARTER, C. M. STEVENS AND L. F. NEY, *J. Biol. Chem.*, 139 (1941) 247.

[18] H. R. V. ARNSTEIN, *Biochem. J.*, 68 (1958) 333.

[19] E. F. JANSEN, *J. Biol. Chem.*, 176 (1948) 657.

[20] D. B. MELVILLE, S. EICH AND M. L. LUDWIG, *J. Biol. Chem.*, 224 [illegible]) 871.

INTESTINAL ABSORPTION OF GALACTOSE

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SUMMARY

Galactose absorption from the rat intestine was studied in the intact animal, in the perfused intestine and in the isolated intestinal loop. In the intact animal an average porto-arterial difference of 16.1 milligram per cent galactose was found. At the same time there was no porto-arterial difference in the total reducing sugar.

In the perfused rat intestine, the entire amount of galactose removed from the intestinal lumen could be recovered in the mucosa and the perfusion fluid. In this case too, no additional reducing sugar appeared in the portal blood. The galactose replaced an equivalent amount of glucose. The presence of glucose in the perfusion fluid was an important factor in galactose absorption; its omission decreased galactose transport into the blood by 40–50%.

Glucose also accelerated galactose transport in the isolated intestine when added to the outer solution, but caused inhibition when introduced into the lumen. Acetate, succinate and pyruvate, up to the concentration of 50 μmoles/ml, were without effect. Higher concentrations of pyruvate accelerated galactose transport to the same extent, irrespective of the site of application.

A working hypothesis is suggested for the resorption mechanism of galactose and other non-metabolized sugars. The relation of this mechanism to that of glucose absorption is discussed.

INTRODUCTION

In the active absorption of glucose from the intestine, the transport of glucose proper across the intestinal cells into the portal blood was supposed by HESTRIN-LERNER AND SHAPIRO[1, 2] to be of only secondary importance. According to their assumption, the bulk of the sugar, taken up from the intestinal lumen, is metabolized in the intestinal cells and metabolic intermediary products are transported into the portal blood. This assumption was based on the following findings: (a) Neither in experiments *in vivo* nor in the perfused intestine did the glucose concentration in the portal blood exceed that in the arterial blood, when low concentrations of glucose (0.2–0.4%) were being absorbed. (b) When [^{14}C]glucose was introduced into the lumen, non-glucose radioactive compounds appeared in the portal blood in concentrations markedly exceeding those in the arterial blood. (c) The rate of glucose metabolism of the intestine was found to be of very high order, and complete combustion of glucose to CO_2 was only a minor factor in the disposition of metabolized glucose.

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This assumption was recently challenged by several investigators[3,4], who showed that during the resorption of [^{14}C]glucose from the intestine the bulk of the radioactivity (80–90 %) in the blood, perfusing the intestinal loop, was associated with glucose and only 10–20 % was found in the form of metabolites like lactic acid, alanine and others. This was considered as proof for the thesis that glucose *per se* is transferred into the portal blood. Moreover, it was shown by TAYLOR AND LANGDON[5], that, following feeding of [1-^{14}C]glucose, liver glycogen had 76–91 % of its radiocarbon in the C_1 position. This was taken to mean that the major part of the glucose was not split into 3-carbon fragments during absorption, since this would have caused a redistribution of the label.

In all these cases, the net transport of glucose was not measured or considered unmeasurable. When repeating the experiments of HESTRIN-LERNER AND SHAPIRO[1] and those of the authors cited above[3,4] it was found by us that all these observations could be confirmed and verified. Thus we were left with the result that, while even with 1 % glucose in the lumen no or negligible portal-arterial differences in glucose could be found, a considerable amount of radioglucose entered the blood from the intestinal lumen.

This discrepancy between the isotopic and chemical results could be explained if one assumed the existence of an exchange of glucose between the mucosal cells and the blood. To test this assumption it was considered of interest to ascertain whether such an exchange exists between mucosal galactose and portal blood glucose. Such an exchange would serve as a possible mechanism for the resorption of galactose and other sugars which are actively absorbed though not metabolized, and at the same time would furnish an explanation of the above stated discrepancy between isotopic and chemical measurements of glucose.

MATERIALS AND METHODS

The experiments were carried out by techniques similar to those in previous reports[1,2] with some modifications as indicated in the following: Rats of 150–200 g were used. All operations were carried out under anaesthesia with pento-barbiturate (5 mg/100 g).

Total reducing sugar was determined according to the method of SOMOGYI AND NELSON[6].

Galactose was determined as the reducing sugar which remained after glucose was removed by glucose-oxidase. 5 ml of the deproteinized solution were incubated together with 0.3 ml of a 5 % solution of the crude enzyme (Notatin) and 0.05 ml 0.2 M phosphate buffer pH 6 for 3 h at 37°. The solution was deproteinized with $ZnSO_4$ (5 %) and $Ba(OH)_2$ (N/3) and the reducing sugar in the filtrate was estimated.

Paper chromatography was developed with butanol–pyridin–water (45:25:40). The chromatogram was run for 36 h, dried and scanned for radioactive spots and then sprayed with aniline–oxalate.

[1-^{14}C]galactose was obtained from the National Bureau of Standards, U.S.A.

RESULTS

Porto-arterial differences in sugar concentrations during galactose absorption from the intestine in vivo

When galactose was introduced into the intestine and portal and arterial blood samples were analyzed, it was found that galactose appeared in the portal blood in concentrations which exceeded those in the arterial vessels markedly and consistently.

The mean porto-arterial difference was 16.1 mg% galactose, a value which may account for the bulk of the galactose disappearing from the intestinal lumen.

However, at the same time, no porto-arterial difference was found in the total reducing sugar concentration, indicating that the galactose which entered the blood replaced an equivalent amount of blood glucose (Table I).

TABLE I

PORTAL ARTERIAL DIFFERENCE IN SUGAR CONCENTRATIONS DURING GALACTOSE RESORPTION

6 ml of a solution containing 50–60 mg galactose in $M/15$ Na_2HPO_4 was injected into the lumen of the small intestine and tied off at both ends. The body cavity was closed and kept warm for 10 min. Blood samples were then extracted simultaneously from the portal vein and from the aorta. Galactose and total reducing sugar were determined in the contents of the lumen, the intestinal tissue and in the blood samples.

mg galactose absorbed	*mg % of total reducing sugar*		*mg % of galactose*		*mg % portal arterial difference**	
	In aorta	*In porta*	*In aorta*	*In porta*	*In total reducing sugar*	*In galactose*
19	126	126	28	44	— 11	+ 12.5
18.7	102	106	26	54	+ 8	+ 30.1
20.3	104	104	24	32	— 5	+ 6.4
17	110	110	24	42	0	+ 18
14.2	120	120	28	44	— 6	+ 13.8

* The portal arterial differences were corrected for shift of water as determined by the haematocrit of portal and arterial blood.

Galactose absorption from the perfused intestine

When the small intestine was perfused *in situ* with artificial blood and a 1% galactose solution was injected into the intestinal lumen, it was again found that galactose could be detected in the perfusion fluid in amounts approaching those disappearing from the intestinal lumen. Here too galactose replaced an equivalent amount of glucose, as no increase in total reducing sugar was found (Table II).

It could furthermore be shown that galactose is transported unchanged and is not metabolized by the intestinal cells. [1-^{14}C]galactose was introduced into the lumen. The intestine was then perfused with artificial blood, and the entire blood leaving the intestine during the absorption period of 30 min was collected.

All of the [1-^{14}C]galactose which disappeared from the lumen could be recovered in the intestinal wall and in the blood without any change in specific activity. Chromatographic analysis confirmed the above assumption: All of the radioactivity was confined to a single spot identified as galactose (Table III).

If the exchange between blood glucose and intestinal galactose was a factor in galactose absorption, the presence or absence of glucose from the perfusion fluid would be expected to influence the rate of galactose absorption. This was actually found to be the case. When the absorbing intestine was perfused with a fluid to which no glucose was added, the rate of absorption was decreased to 40–50% of that when glucose was added. It should be stated that in no case did we obtain a perfusion with a fluid completely free of glucose. Some admixture of glucose from the test animal could not be prevented. In spite of that a considerable increase in absorption was obtained when glucose was added to the perfusion fluid (Table II).

TABLE II

GALACTOSE ABSORPTION IN THE PERFUSED RAT INTESTINE IN THE PRESENCE AND ABSENCE OF GLUCOSE FROM THE PERFUSION FLUID

Perfusion was carried out as described in a previous publication[1]. The perfusion fluid in these experiments consisted of two parts of bovine erythrocytes previously washed 5–6 times with Ringer solution dissolved in three parts of 5% bovine albumin (Pentex) in Ringer solution. The pH was adjusted to 7.4 with 6.5% $NaHCO_3$. Glucose was added to a final concentration of approx. 100 mg %. The fluid was saturated with O_2–CO_2 (95:5) and was warmed to 40° by passing through a coil which was placed in a thermostatic water bath. 50–60 mg galactose in 6 ml of $M/15$ Na_2HPO_4 was injected into the lumen of the intestine. The portal blood was collected in ice cooled tubes during the resorption period which was 10 min.

Blood flow (ml/min)	*Reducing sugar concentration in mg %*		*Galactose concentration in portal blood in mg %*	*mg galactose absorbed**
	In perfusion blood	*In portal blood*		
		With glucose in perfusion fluid		
4.5	86	88	20	15
6	86	88	23	12
5.4	86	88	25	10
3.4	106	96	16	12
6	94	96	12	10
6	90	98	28	19
9	88	90	16	19
4.7	94	90	22	9
4	94	88	16	6.3
1.5	98	76	22	3.2
2.5	74	68	15	4.5
2.3	96	86	27	4
		Mean	20.16 ± 2.19	10.33 ± 1.39
		Without glucose in perfusion fluid		
2	0	4	4	3
5	0	30	12	13
3.5	0	22	12	4
1.5	0	32	18	5
3	2	26	13	4
4.5	2	24	11	5
3	1.5	14	14	5
2.8	2	12	12	3
1.7	0	20	13	4
		Mean	12.11 ± 1.45	5.11 ± 0.25

* Calculated from the amount of galactose which disappeared from the lumen minus the amount retained by the intestinal tissue.

Absorption of galactose from an intestinal loop in vitro

Further evidence for an exchange between glucose and galactose was obtained in experiments with the isolated intestine. When the galactose was introduced into isolated small intestine and this was placed into a tyrode bath, it was found that the penetration of galactose from the lumen into the outer fluid was increased markedly by the presence of glucose in the outer solution. This effect of glucose increased with increasing glucose concentrations up to 55 μmoles/ml. Beyond this concentration the rate of galactose absorption decreased (Table IV).

TABLE III

ISOTOPIC AND CHEMICAL DISTRIBUTION OF [1-^{14}C]GALACTOSE IN THE LUMEN, INTESTINAL TISSUE AND PORTAL BLOOD DURING GALACTOSE RESORPTION

Perfusion was carried out as described in Table II but with [1-^{14}C]galactose in the lumen and 0.1 % glucose in the perfusion fluid.

Isotopic distribution				
Counts/min in lumen before resorption	*Counts/min/after 30 min resorption*			
	In lumen	*In intestinal tissue*	*In portal blood*	*Total*
825,000	583,500	114,000	159,760	857,260
546,000	300,000	50,000	152,000	502,000
592,000	821,000	52,000	208,000	541,000

Chemical distribution				
mg galactose in lumen before resorption	*mg galactose after 30 min resorption*			
	In lumen	*In intestinal tissue*	*In portal blood*	*Total*
50.7	33.1	5.8	9.9	50.8
51	32	4.4	13.6	50
49.8	28.2	5.2	16	49.4

TABLE IV

ABSORPTION OF GALACTOSE FROM AN INTESTINAL LOOP *in vitro* IN THE PRESENCE OF GLUCOSE IN THE OUTER FLUID

Resorption from intestinal loop was studied as reported before[2]. The loop was filled with a solution of 1 % galactose in tyrode, tied at both ends and placed in a beaker containing 50 ml of tyrode solution at 37°. The outer solution contained initially glucose at various concentrations or no glucose at all. CO_2–O_2 (5:95) was bubbled through the outer fluid. Numbers in brackets present the number of the experiments. P was determined according to "Student's Method" in relation to the means of the experiments without glucose.

	Glucose concentration in the outer fluid in µmoles/ml				
	0	*11*	*27*	*55*	*110*
Mg galactose in the outer fluid after 10 min	1.3 ± 0.2 (9)	2.6 ± 0.1 (9) $P < 0.01$	3.4 ± 0.3 (3) $P < 0.01$	6.8 ± 0.5 (5) $P < 0.01$	14 ± 1 (3) $P < 0.01$
Mg galactose in the outer fluid after 20 min	3.8 ± 0.2 (9)	4.4 ± 0.3 (9) $P = 0.1$	6 ± 0.9 (3) $P < 0.05$	8 ± 0.3 (9) $P < 0.01$	15 ± 1 (3) $P < 0.01$

It was further tested whether the activating effect of glucose was due to the fact that it serves as a metabolite of the intestine. Glucose was therefore replaced by other metabolites, like pyruvate, succinate and acetate. The two latter compounds had no effect whatsoever (Table V). Pyruvate was also without effect when tested at concentrations up to 50 µmoles/ml, although these concentrations sustain maximum respiratory rate (Table VI). Only when the pyruvate concentration was raised above 50 µmoles/ml did a significant effect on galactose transport appear.

TABLE V

ABSORPTION OF GALACTOSE FROM AN INTESTINAL LOOP *in vitro* IN THE PRESENCE OF PYRUVATE, SUCCINATE AND ACETATE IN THE OUTER FLUID

The experiments were carried out as described in the heading to Table IV. Figures in the table present µmoles galactose transported into the outer fluid after 10 min.

Metabolite	*Concentration of metabolite in outer fluid in µmoles/ml*				
	0	*10*	*20*	*50*	*100*
Sodium pyruvate	1.3 ± 0.2 (9)	1.6 ± 0.1 (5) P = 0.5	2 ± 0.5 (5) P = 0.2	2.6 ± 0.1 (7) P < 0.01	3.8 ± 0.4 (5) P < 0.01
Sodium succinate	1.3 ± 0.2 (4)	2.1 ± 0.2 (4) P = 0.05	1.9 ± 0.2 (5) P > 0.05	2 ± 0.2 (4) P = 0.05	1.6 ± 0.7 (3)
Sodium acetate	1.3 ± 0.2 (9)	0.9 ± 0.2 (2)	1.2 ± 0.3 (3)	1.5 ± 0.2 (4)	

TABLE VI

RESPIRATION OF INTESTINAL TISSUE IN THE PRESENCE OF PYRUVATE

About 100 mg of fresh tissue was introduced into small Warburg vessels containing Krebs–Ringer solution with pyruvate at the indicated concentrations, with 10% KOH and filter paper in the centre well.

Concentration of pyruvate µmoles/ml	*µl O_2 consumed/h/100 mg fresh tissue*
0	27
10	67
20	57

TABLE VII

ABSORPTION OF GALACTOSE FROM AN INTESTINAL LOOP *in vitro* IN THE PRESENCE OF GLUCOSE, PYRUVATE AND SUCCINATE IN THE LUMEN

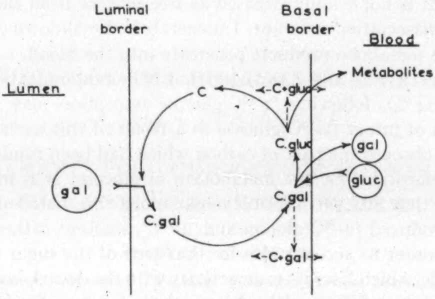
The experiment was carried out as described before[2]. Glucose, pyruvate and succinate at various concentrations were introduced together with galactose (55 µmoles/ml) into the inner solution. Galactose in the outer solution was determined after 20 min.

Addition		*mg galactose transported*
To outer fluid	*To inner fluid*	
—	—	3.8 ± 0.2
Glucose 27 µmoles/ml	—	6.0 ± 0.9
—	Glucose 27 µmoles/ml	3.8 ± 0.4
Glucose 55 µmoles/ml	—	8.0 ± 0.3
—	Glucose 55 µmoles/ml	2.1 ± 0.28
Pyruvate 20 µmoles/ml	—	4.4 ± 0.4
—	Pyruvate 20 µmoles/ml	4.7 ± 0.6
Pyruvate 50 µmoles/ml	—	5.4 ± 0.2
—	Pyruvate 50 µmoles/ml	4.8 ± 0.3
Succinate 20 µmoles	—	4.0 ± 0.5
—	Succinate 20 µmoles/ml	4.3 ± 0.2
Succinate 50 µmoles	—	5.8 ± 0.3
—	Succinate 50 µmoles/ml	4.3 ± 0.3

Glucose and pyruvate differ in their behaviour towards galactose absorption in another sense, in addition to the quantitative differences. While pyruvate exerted the same effect whether this substance was applied at the serosal or mucosal side (Table VII), the glucose effect showed a marked polarity, *i.e.* addition to the mucosal side together with the galactose caused inhibition of galactose uptake, as has been repeatedly shown[7–10].

DISCUSSION

The results presented in the present paper lead us to the postulation of the following scheme for galactose absorption:



The galactose which penetrates the mucosal cells from the lumen is reversibly bound to a carrier (c). This carrier–galactose complex migrates to the serosal side and there, glucose derived from the blood competes with galactose for the carrier and galactose is replaced by glucose and migrates into the extracellular space and portal blood. The carrier–glucose complex is decomposed by the metabolism of glucose and removal of the metabolic products to the blood stream. The carrier is thereby liberated and can combine at the mucosal side with an additional molecule of galactose.

This scheme is based on the following considerations:

The assumed exchange between galactose and glucose is corroborated by the finding that, in all levels of experiments, the total reducing sugar in the blood or bathing solution remained unchanged, while galactose replaced part of the glucose. Furthermore, blood glucose was shown to accelerate galactose transport. The assumption that both sugars combine with the same carrier is based on the finding presented here and made previously by others[7–10] that glucose competes with galactose when both sugars are introduced together into the lumen. In these experiments, FISHER AND PARSONS could determine a Michaelis constant for the resorption process, which was $8.3 \cdot 10^{-3}$ for glucose[11] and $3.5 \cdot 10^{-2}$ for galactose[9]. These constants may be actually the dissociation constant of the sugar–carrier complexes as postulated above. The lower dissociation constant of the glucose than that of the galactose-carrier complex makes glucose an efficient exchanging agent. Just as glucose competes with galactose for the carrier when introduced into the lumen, it replaces galactose from the carrier at the blood border and thereby increases the concentration of free galactose at this site and causes an acceleration in the transfer of galactose into the blood. The scheme would thus explain the polarity of the glucose effect, demonstrated here, competing at the luminal border and accelerating at the blood border.

The proposed mechanism for galactose absorption may be a more general one, facilitating the transfer of all compounds which have affinity for the carrier, *i.e.* show certain structural similarities with glucose and galactose[12–14]. It seems also to be the basis for the penetration of radioactive glucose into the portal blood, without a corresponding net increase in glucose concentration. The results presented demonstrate that it is technically possible to detect net increase in the portal blood of a substance which is absorbed at the rate of galactose and of glucose, provided that it gets into the blood unchanged.

Glucose differs from the other sugars in being not only bound by the carrier but also metabolized. It is not readily released as free glucose from the carrier complex, because of its low dissociation constant. The metabolic breakdown of glucose liberates the carrier and the metabolic products penetrate into the blood.

The findings of TAYLOR AND LANGDON[5] that little randomization occurs in ^{14}C in liver glycogen (9–24%), following [1-^{14}C]glucose resorption may also be explained by the penetration of intact [1-^{14}C]glucose as a result of this exchange process. This penetration would obscure the part of carbon which had been randomized due to the main process of resorption, *i.e.* the metabolism of glucose. It is interesting to note, in this connection, that SIU AND WOOD[15] re-examined this distribution of ^{14}C in liver glycogen. They introduced [2-^{14}C]glucose and [2-^{14}C]galactose, rather than the C_1 labelled compounds, in order to account also for that part of the sugar which is degraded *via* the pentose cycle, which loses its radioactivity with the decarboxylation of C_1. With these markers they found that with glucose there was randomization of 20–30% whereas with galactose this was less than 10%. This difference was partly attributed to the fact that glucose is degraded in the intestine to a considerable extent while galactose is not.

The nature of the metabolic products of glucose has been shown to be lactic acid[3,4,16–18]. Other compounds also seem to appear in smaller amounts. The results of our own experiments varied and smaller or larger amounts of lactic acid were found. The exact nature of the products seems to depend on the conditions, as suggested by ATKINSON *et al.*[4].

It should further be stated that WILSON AND WISEMAN[16] and WILSON AND VINCENT[19] could show with the aid of everted hamster intestine a considerable capacity to concentrate glucose and galactose against a concentration gradient. This would point to a mechanism different from that suggested here. It remains to be settled what the relative importance of these various mechanisms is. It is quite possible that in this respect there exist marked species differences, the mechanism suggested here predominating in the rat and the active concentration of glucose in the hamster.

REFERENCES

[1] S. HESTRIN-LERNER AND B. SHAPIRO, *Biochim. Biophys. Acta,* 13 (1954) 54.
[2] S. HESTRIN-LERNER AND B. SHAPIRO, *Biochim. Biophys. Acta,* 12 (1953) 533.
[3] J. Y. KIYASU, J. KATZ AND I. L. CHAIKOFF, *Biochim. Biophys. Acta,* 21 (1956) 286.
[4] R. M. ATKINSON, B. J. PARSONS AND D. H. SMYTH, *J. Physiol. (London),* 135 (1957) 581.
[5] W. R. TAYLOR AND R. G. LANGDON, *Biochim. Biophys. Acta,* 21 (1956) 385.
[6] M. SOMOGYI, *J. Biol. Chem.,* 195 (1952) 19.

[7] C. F. Cori, *Proc. Soc. Exptl. Biol.*, 23 (1926) 290.
[8] H. G. K. Westenbrink and A. Middlebeck, *Arch. néerl. physiol.*, 21 (1936) 283.
[9] R. B. Fisher and D. S. Parsons, *J. Physiol.* (*London*), 119 (1953) 224.
[10] E. Riklis, B. Haber and J. H. Quastel, *Can. J. Biochem. and Physiol.*, 36 (1958) 373.
[11] R. B. Fisher and D. S. Parsons, *J. Physiol.* (*London*), 119 (1953) 210.
[12] P. N. Campbell and H. Davson, *Biochem. J.*, 43 (1948) 426.
[13] R. K. Crane and S. M. Krane, *Biochim. Biophys. Acta*, 20 (1956) 569.
[14] T. H. Wilson and R. K. Crane, *Biochim. Biophys. Acta*, 29 (1958) 30.
[15] P. M. L. Siu and H. G. Wood, *J. Biol, Chem.*, 234 (1959) 2223.
[16] T. H. Wilson and G. Wiseman, *J. Physiol.* (*London*), 123 (1954) 116.
[17] T. H. Wilson, *J. Biol. Chem.*, 222 (1956) 751.
[18] H. Newey, D. H. Smyth and B. C. Whaler, *J. Physiol.* (*London*), 129 (1955) 1.
[19] T. H. Wilson and T. N. Vincent, *J. Biol. Chem.*, 216 (1955) 851.

STUDIES ON THE BIOLUMINESCENCE OF *RENILLA RENIFORMIS*

I. REQUIREMENTS FOR LUMINESCENCE IN EXTRACTS AND CHARACTERISTICS OF THE SYSTEM*

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SUMMARY

A method is described for the preparation of luminescent extracts from the sea pansy, *Renilla reniformis*. Light production requires the presence of *Renilla* luciferase, an adenine-containing nucleotide (either ATP, ADP, or AMP), *Renilla* luciferin, and oxygen. The reaction is specific for adenine-containing nucleotides and *Renilla* luciferin. The luminescent reaction was shown to involve a nucleotide-dependent oxidation of *Renilla* luciferin.

Various factors that affect the system were studied. These include the effect of pH, enzyme concentration, temperature, inhibitors, nucleotide concentration, and luciferin concentration.

The evidence presented indicates that ATP is involved indirectly in this sytem by functioning as a generating system for ADP and AMP via an ATPase reaction.

Abbreviations used: AMP, ADP, and ATP are the mono-, di- and triphosphates of adenosine respectively; CMP, CDP and CTP are the mono-, di- and triphosphates of cytidine respectively; UMP, UDP, and UTP are the mono-, di- and triphosphates of uridine respectively; GMP, GDP, and GTP are the mono-, di- and triphosphates of guanosine respectively; GSH, reduced glutathione; PCMB, *p*-chloromercuribenzoate; ATPase, adenosine-5′-triphosphatase; FMN, $FMNH_2$, flavin mononucleotide and dihydroflavin mononucleotide respectively; FAD, flavin adenine dinucleotide.

* This is contribution No. 18 from the University of Georgia Marine Institute, Sapelo Island, Georgia.

INTRODUCTION

Although many thousands of bioluminescent species exist throughout the animal kingdom[1], our knowledge of the biochemistry of these light reactions is scanty. Studies on the identity of factors required for the luminescence of extracts of luminous bacteria, the firefly, and the luminous ostrocod, *Cypridina*, have been reported in recent years[2-3]. Data on one additional luminescent system, the luminous fungi, has recently been reported[4]. With the exception of the four systems mentioned above, the nature of the components involved in other bioluminescent systems has not been established. This report is the first of a series of studies that deals with the luminescence system of the sea pansy, *Renilla reniformis*, and represents the first description of luminescence in extracts of this organism. The requirements for luminescence in extracts are presented as well as additional characteristics of the system. A preliminary report of a portion of this work has appeared[5].

MATERIALS AND METHODS

The purine and pyrimidine nucleotides and the adenosine used in these experiments were obtained from the Sigma Chemical Company.

Preparation of crude extracts

Freshly collected sea pansies were placed in a dark, running sea water aquarium for 4 to 8 h. Since these animals have a diurnal rhythm of luminescence[1], this procedure was necessary to rejuvinate the luminescence system. Approximately 50 animals at a time were destemmed and dropped into a cold mortar containing 10 ml saturated ammonium sulfate, pH 7.5. They were cut into small pieces with scissors and finally ground with sand for 30 min. A total of 200 ml of saturated ammonium sulfate was gradually added with stirring and the suspension centrifuged at 12,000 × *g* for 10 min. The precipitate was carefully drained to remove excess ammonium sulfate, and then extracted by grinding in a mortar with a total of 50 ml of 0.05 *M* potassium phosphate buffer, pH 7.5, containing 0.001 *M* GSH(phosphate–GSH buffer). The extract was centrifuged for 10 min at 12,000 × *g* and the resulting supernatant centrifuged for 30 min at 140,000 × *g*. The latter centrifugation yields a clear, amber colored supernate which contains the activity and will be referred to as the crude extract. The activity of the extract was preserved by storing at —20°. The grinding, centrifugation and extraction procedures were done at 0–3°.

For the experiments reported here, unless otherwise indicated, the crude extract was precipitated once with saturated ammonium sulfate, pH 7.5 (to 75 % saturation), and the precipitated protein redissolved in phosphate–GSH buffer.

Treatment of the animals with ammonium sulfate is an essential feature of the extraction process. Grinding rejuvinated organisms with sand in the presence of phosphate–GSH buffer without ammonium sulfate treatment does not yield an active extract. Ammonium sulfate produces at least two observable effects: firstly, it allows a greater amount of protein to be extracted; and secondly, it acts as an anesthetic thus preventing an excessive luminescent response during grinding, which would result in oxidation of *Renilla* luciferin.

Preparation of crude Renilla *luciferin*

The once extracted *Renilla* residue was reextracted with phosphate–GSH buffer in the same manner as described for preparation of the crude extract except that centrifugation at 140,000 × *g* was not required. This extract was heated at 100° for 1.5 min, cooled to room temperature, and the denatured protein removed by centrifugation. The supernate contained *Renilla* luciferin which can be stored at —15° for weeks without significant loss of activity.

Assay system

Unless otherwise specified, the luminescence assay system consisted of the following additions: potassium phosphate buffer, pH 7.5 (50 µmoles); GSH(2 µmoles); ATP, ADP, or AMP(5 µmoles); *Renilla* luciferin (0.2 ml); enzyme (0.5 mg protein); water to 1.5 ml. In the experiments reported, ADP was used in preference to ATP since the latter compound functions indirectly in this system. All reactions were carried out at 30° unless otherwise stated.

Light measurements

Light from this luminescence system was measured by a dry-ice cooled 1P21 photomultiplier made by RCA. Approx. 960 V was applied across the photomultiplier by a Victoreen, model 683, DC power supply. The output of the photomultiplier was channeled into a Keithley, model 410, micro-microammeter and the output of the latter channeled into a Varian, model G-10, recorder. This system is quite sensitive and allows continuous recording of luminescence with time.

Temperature effects

The effect of temperature on luminescence was studied over the temperature range of 5–40°. The procedure followed is the same as that followed by STREHLER AND CORMIER during similar studies on the bacterial luminescence system[12] and tends to eliminate systematic errors due to enzyme denaturation or transient changes of other types.

RESULTS

Characteristics of Renilla *luciferase*

Crude extracts from *R. reniformis* give a relatively weak luminescence that is stimulated approximately 10–30 fold (depending upon the extract) on the addition of either ATP, ADP, or AMP. These results are presented in Table I. Enzyme preparations can be obtained, by repeated precipitations with saturated ammonium sulfate, that are stimulated 100 fold upon the addition of adenine-containing nucleotides. Adenosine is inactive in this system, as well as the monophosphates and tri-phosphates of guanosine, cytidine and uridine. The diphosphates of guanosine and cytidine are also inactive although some activity was exhibited by UDP. Whether the latter activity is due to contamination with adenine-containing nucleotides is not certain, although on the basis of concentration curves (Fig. 1), 1 % contamination with any one, or a combination, of the adenine-containing nucleotides would have been sufficient to account for the light observed. Furthermore, since UTP and UMP are inactive, it appears unlikely that uridine-containing nucleotides are active in this system. Thus, luminescence in extracts of *R. reniformis* is dependent upon the presence of adenine-containing nucleotides and is specific for the adenine moiety, at least in the initial step of the reaction. The enzyme responsible for light emission in these extracts will be referred to as *Renilla* luciferase.

TABLE I

EFFECT OF NUCLEOTIDES ON THE LUMINESCENCE OF *R. reniformis* EXTRACTS

Conditions: Same as described in the MATERIALS AND METHODS section. 5 μmoles of each nucleotide or nucleoside were added as indicated.

Expt.	*Addition*	*Relative light intensity*
I	None	16
	ATP	445
	ADP	390
	AMP	440
	Adenosine	16
	GTP, CTP, or UTP	16 ± 1
	GMP, CMP, or UMP	16 ± 1
II	None	20
	ADP	450
	GDP	23
	CDP	36
	UDP	122

Since light emission in the firefly requires a primary activation by ATP[14], and, since the *Renilla* system responds to either ATP, ADP or AMP, there appeared to be a good possibility that the two systems were similar with respect to nucleotide requirements. The purity of the ADP and AMP was therefore checked for contamination with ATP. The AMP used in these experiments did not contain ATP as determined by ATP analysis using the firefly technique[2,6]. The sensitive light detecting apparatus used in these experiments readily allowed the detection of 10^{-3} μg ATP/ml. Likewise, insufficient amounts of ATP were found in the ADP preparations to account for the light observed. Furthermore, various commercial sources of ADP and AMP were found to be equally effective. In addition, the AMP used was not contaminated with ADP, which was measured by coupling adenylate kinase to the firefly system. The activities exhibited by AMP and ADP, therefore, were not due to contamination with ATP.

The possibility existed, however, that the AMP and ADP activities could be attributed to phosphorylations in the crude extract leading to the formation of ATP. This possibility can be ruled out since AMP and ADP are equally effective in a phosphate-free system as well as in the presence of 2,4-dinitrophenol (10^{-2} *M*, final concentration). The method of FISKE AND SUBBAROW[7] was used to determine the absence of inorganic phosphate in these experiments. In addition, adenylate kinase could not be demonstrated in the crude extracts since, under anaerobic or aerobic conditions, no ATP formation could be detected from ADP using the firefly assay. Thus, the AMP and ADP activities are not due to contamination with ATP or to their conversion to ATP.

At this point it is significant to note that crude extracts contain a phosphatase(s) that causes the liberation of Pi from ATP and ADP. In one experiment, for example, it was found that a preparation containing 1.5 mg protein/ml caused the release of 0.5 and 1.0 μmole of Pi/10 min from ADP and ATP respectively under the experimental conditions given in MATERIALS AND METHODS. Under conditions that support luminescence then, these extracts apparently convert ATP to ADP, whereas ADP is apparently converted to AMP. That these products are actually formed via phosphatase action was confirmed by separation and identification of the nucleotides on formate-charged Dowex-1 columns, as described by Hurlbert *et al.*[8]. The fact that ATP can be broken down to ADP and AMP in the crude extracts, whereas AMP or ADP are not converted to ATP, suggest that ATP functions as a generating system for ADP and AMP and that one (or both) of the latter two nucleotides is directly responsible for driving the light reaction. This suggestion is supported by additional data included later in the text.

The effect of nucleotide concentration on the luminescence of *Renilla* extracts is illustrated in Fig. 1. The data in Fig. 1 show that ATP is more effective than ADP or AMP at the lower concentrations, and that the response obtained with ADP is somewhat less than that with AMP. The higher efficiency of ATP at lower concentrations is also shown by calculation of the apparent Michaelis constants, which, for the ATP reaction, is approximately one-fifth that of the ADP or AMP stimulated reactions. Since generating systems are frequently more efficient than substrate levels of a reactant, the higher efficiency of ATP could be explained on the basis that it acts as a generating system for ADP and AMP.

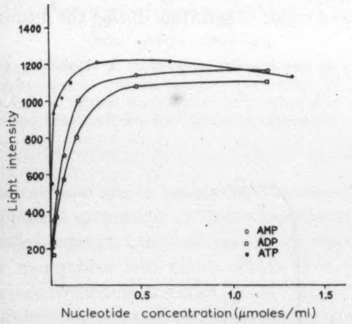

Fig. 1. Effect of nucleotide concentration on the luminescence of *Renilla reniformis* extracts. Conditions are the same as described under MATERIALS AND METHODS except that the nucleotides were added as indicated.

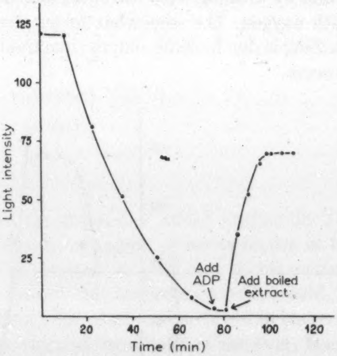

Fig. 2. Effect of boiled extract on luminescence after luminescence decay. Conditions are the same as described under MATERIALS AND METHODS with the exception that ADP and boiled extract were added as indicated.

A requirement for metal ions could not be demonstrated regardless of the nucleotide added. This was true even after repeated ammonium sulfate precipitations followed by dialysis. The lack of a metal requirement in the case of ATP is surprising if the ATP effect is to be explained by ATPase activity. It is not unusual, however, to have difficulty showing metal requirements for this type reaction in relatively crude extracts.

Since other bioluminescence systems require an oxidizable substrate, termed luciferin, for luminescence, an attempt was made to demonstrate the necessity for luciferin in this system. The requirement for an additional factor became apparent by studying luminescence decay as a function of time. Fig. 2 shows that, at the end of approximately 70 min, the luminescence rate decays to a negligible value that is not affected by the addition of nucleotides. Although ADP is added in this case, ATP and AMP are also without effect. Nucleotides are required, however, to bring about the luminescence decay. For example, an extract incubated 70 min in the absence of nucleotides responds to nucleotide addition in a manner analogous to non-incubated extracts. The addition of boiled extracts promotes a rapid return to a steady state value, which depends upon the amount of boiled extract added. Thus, the decay of luminescence is brought about as the result of a nucleotide-dependent utilization of some unknown factor required for luminescence. The factor in the boiled extract, which was been found to be dialyzable, will be referred to as *Renilla* luciferin by analogy to other bioluminescence systems.

Oxygen, as well as nucleotides, is required for luciferin utilization, since a decrease in luminescence activity does not occur by incubation of the complete system under anaerobic conditions. The effect of oxygen on the luminescence of *Renilla* extracts is illustrated in Fig. 3. Apparently, normal oxygen tension is not sufficient to saturate this system, since flushing with oxygen causes an increase in the steady state luminescence rate whereas flushing with air does not. The light is essentially abolished in 2 min by flushing with nitrogen, and the signal is returned by subsequently flushing with oxygen. The somewhat lower steady state value following the second oxygen flushing is due to some enzyme inactivation as a result of agitation during the flushing process.

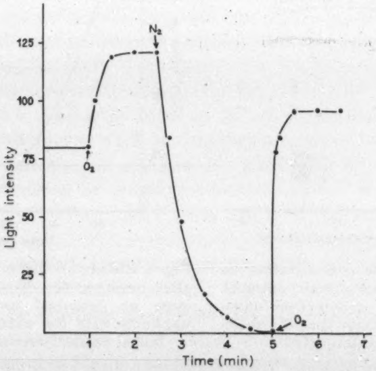

Fig. 3. Effect of oxygen on luminescence. Conditions are the same as described under MATERIALS AND METHODS except that the system was flushed alternately with oxygen and nitrogen as indicated.

Thus the luminescent reaction involves a nucleotide-dependent oxidation of luciferin. Indeed, some preliminary observations on the properties of luciferin indicate that it is oxidizable under certain conditions. For example, luciferin activity is destroyed by heating at 100° for 1 min in the presence of 0.06 N HCl and oxygen, but is stable at this temperature and HCl concentration providing the system is kept anaerobic.

An enzyme system whose luminescence has been allowed to decay to a negligible level in the presence of nucleotide can be treated with ammonium sulfate to precipitate the enzyme and the latter (resolved enzyme) used as an assay for the unknown factor. When this is done, the effect of increasing concentrations of boiled extract on the light intensity can be studied. The results in Fig. 4 show that 80-fold stimulations of luminescence were observed upon the addition of sufficient amounts of crude luciferin.

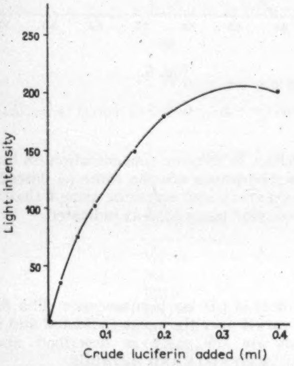

Fig. 4. Effect of luciferin concentration on luminescence. Conditions are the same as described under MATERIALS AND METHODS except that luciferin was added as indicated.

TABLE II

EFFECT OF VARIOUS LUCIFERIN PREPARATIONS ON THE LUMINESCENCE OF *Renilla* EXTRACTS

Conditions: Same as described under MATERIALS AND METHODS except that the source of luciferin was that listed in the table, and the enzyme preparation was a resolved enzyme of the type used to obtain the data in Fig. 4.

Source of luciferin preparation added	*Light intensity*
None	0
Renilla	60
Firefly	0
Cypridina	0
Bacterial ($FMNH_2$)	0
Fungal	0

No increase above background is observed in the absence of added nucleotide. The apparent saturation at higher levels may be due to inhibitors of luminescence in the boiled extract. Luciferin cannot be replaced by divalent cations such as magnesium or manganese and many others that were tested. No luciferin activity could be demonstrated by the addition of long chain aldehydes which are involved in bacterial luminescence, yeast concentrates, or of crude extracts from beef or rat liver, heart, kidney or spleen. After each of these additions, the luminescence system would still respond to the addition of boiled *Renilla* extract. Thus *Renilla* luciferin cannot be demonstrated in a variety of naturally occurring materials. Similar observations have also been made in this laboratory for fungal and firefly luciferins.

Luciferin preparations from a number of bioluminescent systems were tested for activity in the *Renilla* luminescence system. Table II shows that firefly*, *Cypridina*, bacterial, or fungal luciferin will not replace *Renilla* luciferin. All luciferin preparations were shown to be active in their respective luminescence systems prior to testing them with *Renilla* luciferase. Likewise, *Renilla* luciferin will not react in any of the

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* The author wishes to express his appreciation for the generous gifts of firefly and *Cypridina* luciferins by Drs. W. D. McELROY and E. N. HARVEY respectively.

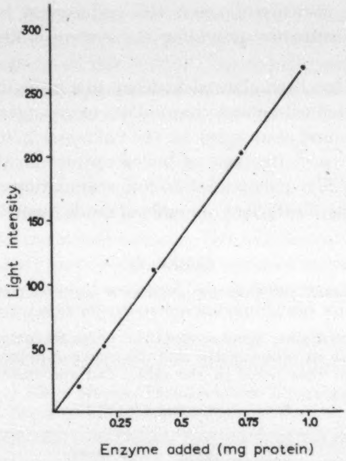

Fig. 5.

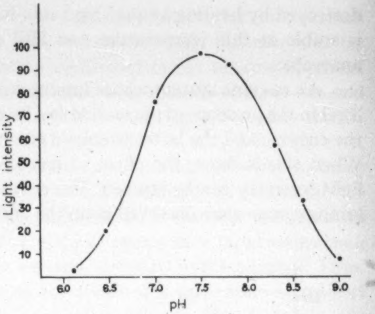

Fig. 6.

Fig. 5. Effect of enzyme concentration on luminescence. Conditions are the same as described under MATERIALS AND METHODS except that the enzyme was added as indicated.

Fig. 6. Effect of pH on luminescence. The final pH was varied over the range indicated and the conditions are the same as described under MATERIALS AND METHODS.

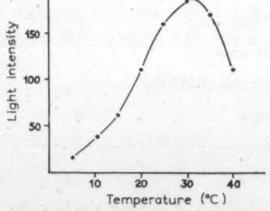

Fig. 7.

Fig. 7. Effect of temperature on luminescence. The temperature was varied as indicated. The procedure followed and the conditions used are the same as that described under MATERIALS AND METHODS.

above mentioned luminescence systems. *Renilla* luciferin is specific, therefore, for *Renilla* luciferase.

Fig. 5 illustrates the effect of luciferase concentration on luminescence. A typical straight line response is obtained with increasing enzyme concentration.

The response of this luminescence system to pH is shown in Fig. 6. The activity declines on the acid side of neutrality, but shows considerable activity over a relatively wide range on the alkaline side with an optimum at pH 7.5.

Fig. 7 illustrates the effect of temperature on luminescence. Under these experimental conditions, the optimum for this reaction is 32°.

Although the spectral energy distribution of the light from this luminescent reaction has not been determined, the luminescence is visible to the 5 min dark-adapted eye, and some visual observations have been made. The greenish-blue color of the light in extracts appears to the eye to be the same color as that emitted from the intact animal.

Inhibitor studies

A number of well known inhibitors of other bioluminescent systems, as well as classical enzyme inhibitors, were tested for their inhibitory action on *Renilla* luminescence. Table III shows that potent inhibitors of bacterial luminescence such as menadione and cytochrome *c*[2,9] do not inhibit luminescence of *Renilla* extracts, indicating that these compounds do not oxidize *Renilla* luciferin as they do bacterial luciferin ($FMNH_2$).

Fluoride is a partial inhibitor at high concentrations, but only when ATP is added. For example, when ADP is used, fluoride does not inhibit luminescence as shown in Table III. Similar results were obtained with AMP. In the presence of ATP, however, a 33 % inhibition is observed. Under the same conditions, a 34 % inhibition of ATPase activity by fluoride was observed. These observations support the suggestion that ATP is involved indirectly in this system via an ATPase reaction.

TABLE III

EFFECT OF INHIBITORS ON *Renilla* LUMINESCENCE

Conditions: Same as listed under MATERIALS AND METHODS. Inhibitors added as indicated.

Addition	*Final concentration (M)*	*Per cent inhibition*
NaF	10^{-1}	0
Menadione	10^{-4}	0
Cytochrome c	10^{-5}	0
KCN	10^{-3}	0
NaN_3	10^{-3}	0
FMN	10^{-5}	17
FAD	10^{-5}	8
Na_3ASO_4	10^{-3}	0
Versene	10^{-3}	58
	10^{-2}	63
Sodium pyrophosphate	10^{-3}	0
	$3 \cdot 10^{-2}$	10
PCMB	10^{-4}	93
N-ethylmaleimide	10^{-4}	31
$CuSO_4$	10^{-4}	100
	10^{-5}	85
$AgNO_3$	10^{-4}	100
	10^{-5}	63

The respiratory inhibitors cyanide and azide are non-inhibitory at the concentrations tested. FMN and FAD are slightly inhibitory at 10^{-5} *M*, whereas arsenate does not inhibit.

It has been shown, in the case of firefly luminescence, that inorganic pyrophosphatase can adversely affect the steady state luminescence[11]. Likewise, inorganic pyrophosphate can either inhibit or stimulate luminescence depending upon whether it is added prior to or after ATP. These results have been explained in terms of a primary ATP-dependent activation reaction that results in the formation of luciferyl adenylate and pyrophosphate[10]. As seen from Table III, inorganic pyrophosphate has little or no effect on *Renilla* luminescence. This is true regardless of the order in which pyrophosphate is added. Furthermore, the addition of inorganic pyrophosphatase (300–600 units) has no effect on luminescence of this system. These observations are in direct contrast to those made by McElroy *et al.*[11] on firefly luminescence, and indicate a difference in the mechanism of ATP action in the two systems. In addition, it would be difficult to explain the stimulation of *Renilla* luminescence by AMP on the basis of identical mechanisms.

A requirement for free sulfhydryl groups in the luminescent system is indicated by the inhibitory action of PCMB, N-ethylmaleimide, and copper and silver salts as shown in Table III. The inhibition produced by PCMB can be partially reversed (to 60 % of the initial response) by the addition of 4 μmoles of GSH. In addition, GSH has been found to help maintain luciferase activity during initial purification steps.

Regardless of the nucleotide used in the assay system, a large number of metal ions have no effect on the activity of the crude extract at 10^{-3} M concentrations. These include magnesium, manganese, nickel, calcium, cobalt, zinc, molybdenum, and iron. Although metal ions have no effect on the crude extract, chelating agents such as Versene are potent inhibitors as seen in Table III. Regardless of the nucleotide used in the test system, the inhibitory action of Versene requires approximately 15 min incubation with the test system to reach a maximum level. The addition of magnesium or manganese ions, in amounts in excess of that of Versene, does not reverse the inhibition. Thus, Versene inhibition cannot be explained simply in terms of binding one of these metal ions.

In the absence of phosphate, zinc ion is a potent inhibitor of luminescence. Under these conditions, for example, luminescence is completely inhibited at $2 \cdot 10^{-3}$ M zinc ion. The lack of zinc inhibition in phosphate buffer is due to the fact that an insoluble zinc phosphate forms at the pH used in the assay system.

Although the luminous bacteria and sea pansies discussed here are both marine organisms, the properties of the enzymes involved in the two luminescent systems differ considerably in their response to salt. For example, salts such as NaCl and KCl inhibit the luminescence system of *Renilla*. The inhibition increases linearly with increasing salt concentration, 50 % inhibition being reached at approximately $2 \cdot 10^{-1}$ M salt. The luminescence of extracts of luminous bacteria, on the other hand, is stimulated by increasing salt concentrations, reaching a maximum steady state at approximately $1.7 \cdot 10^{-1}$ M salt[13].

DISCUSSION

Although ATP is quite active in supporting luminescence in crude extracts of *R. reniformis*, the data presented suggest that ATP functions indirectly via an ATPase reaction. The products of this reaction are ADP and AMP, and one of these must be responsible for supporting luminescence in this system. This suggestion is based on a number of observations. Firstly, the system is specific for adenine-containing nucleotides. Secondly, the AMP and ADP used were not contaminated with ATP nor are they converted to ATP by phosphorylation reactions. In addition, the lack of an adenylate kinase in the extracts ruled out the formation of ATP from ADP. Thirdly, the crude extracts contain ATPase activity that rapidly converts ATP to ADP and AMP. Thus it appears that ATP functions simply as a generating system for ADP and/or AMP.

Other supporting data for the indirect role of ATP in this system involves studies with inhibitors. For example, fluoride will partially inhibit ATP-stimulated luminescence, but has no effect on the ADP or AMP driven reactions. The fact that the crude extracts contain an ATPase that is also partially inhibited by fluoride is consistent with the view that ATP functions as a generating system for ADP and AMP via an ATPase reaction.

Recent studies on the purification of *Renilla* luciferase in this laboratory have shown that as the ATPase activity is removed, the luminescence response to ATP drops accordingly, while the response to ADP or AMP is unaffected. In addition, recent studies with partially purified fractions show much faster kinetics for ADP and AMP as compared to ATP. These recent observations leave no doubt that ATP plays an indirect role in *Renilla* luminescence. Further studies on the purification of *Renilla* luciferase, and on the mechanism of nucleotide action in this system, are in progress.

The above considerations indicate a distinct difference in the biochemical mechanisms involved in firefly and sea pansy bioluminescence. Whereas firefly luminescence requires a primary activation by ATP, sea pansy luminescence is stimulated indirectly by ATP. The data indicate the direct participation of ADP or AMP, rather than ATP, in the luminescence reaction of the sea pansy.

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REFERENCES

[1] E. N. HARVEY, *Bioluminescence*, Academic Press, New York, 1952.
[2] W. D. MCELROY AND B. L. STREHLER, *Bacteriol. Rev.*, 18 (1954) 177.
[3] F. I. TSUJI, A. M. CHASE AND E. N. HARVEY in F. H. JOHNSON, *The Luminescence of Biological Systems*, American Association for the Advancement of Science, Washington, D.C., 1955, p. 127.
[4] R. L. AIRTH AND W. D. MCELROY, *J. Bacteriol.*, 77 (1959) 249.
[5] M. J. CORMIER, *J. Am. Chem. Soc.*, 81 (1959) 2592.
[6] B. L. STREHLER AND J. R. TOTTER, *Arch. Biochem Biophys.*, 40 (1952) 28.
[7] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[8] R. B. HURLBERT, K. SCHMITZ, A. F. BRUMM AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.
[9] M. J. CORMIER, J. R. TOTTER AND H. H. ROSTORFER, *Arch. Biochem. Biophys.*, 63 (1956) 414.
[10] W. C. RHODES AND W. D. MCELROY, *J. Biol. Chem.*, 233 (1958) 1528.
[11] W. D. MCELROY, J. W. HASTINGS, J. COULOMBRE AND V. SONNENFELD, *Arch. Biochem. Biophys.*, 46 (1953) 399.
[12] B. L. STREHLER AND M. J. CORMIER, *Arch. Biochem. Biophys.*, 53 (1954) 138.
[13] B. L. STREHLER AND M. J. CORMIER, *Arch. Biochem. Biophys.*, 47 (1953) 16.
[14] W. D. MCELROY AND A. GREEN, *Arch. Biochem. Biophys.*, 64 (1956) 257.

Short Communications

Bacteria and their flagella

Flagella are difficult to observe on living bacteria for they have extremely small diameters[1]. Under an electron microscope, however, they appear to be made up of very fine filaments of macromolecular dimensions which are twisted together in rope-like structures[2–4]. How flagella are utilized by bacteria has been debated over the years since they were first seen on fixed and stained preparations. Flagella generally are found on motile bacterial cells and when cells are in motion the flagella, when seen, also appear to be in motion. The circumstantial evidence is strong for the belief that flagella are organs of locomotion[4,5]. On the other hand, there is some indication that they reduce the forward speed because of viscous drag[6].

It has been difficult to conceive the ultrafine flagellar filaments themselves to be sufficiently complex in structure to support metabolic or other processes for producing energy-rich molecules and at the same time to convert the released energy to kinetic energy of locomotion. Some investigators have suggested that the source of energy is not the flagellum but the bacterial cell itself. It is assumed that the bacterium rather leisurely snaps the flagellum as a whip, or coils and uncoils it at different rates, thus propelling itself forward.

Unfortunately, this suggestion also meets with difficulties. Because of viscous drag the flagellum would be unable to support a wave, certainly not one of uniform amplitude[11]. The base end would need to continually execute to and fro displacements equal to the amplitude of the undulations on the flagellum and even then the motion would be damped out so rapidly that there would be no apparent waveform. A piece of flagellum with an initial speed of 30 μ/sec in water, for example, would come to rest within a distance of less than 1 mμ.

A third group of investigators under the leadership of Pijper have suggested that bacterial locomotion is accomplished by the sinuous movements of the bacterium itself and that the flagella are really trails of materials sloughed off a slime layer[7]. In other words, the flagella serve neither as organs nor as propellers for locomotion; they are simply inactive appendages. As pointed out by Houwink and Van Iterson[5], however, the electron-microscopic observations suggest that the high degree of uniformity in cross-section and the attachment of the flagellum to the cell are not consistent with this picture.

In this note we suggest that some means of stabilization is needed by a bacterium if it is to undergo long range translatory motions and that flagella may well serve as such stabilizers.

Translational Brownian motion commonly is observed among bacteria, for they are small enough to reveal the effects of fluctuations in the impacts of molecules of the growth medium. In essence, the bacteria are large "molecules" in thermal agitation. Perhaps not so well known is the phenomenon of rotational Brownian motion. An equation for the mean-square angle of rotation for a spherical particle was derived by Einstein[8] and experimentally verified by Perrin[9]. A similar equation may be deduced for the rotation of a small cylinder about a transverse axis through its center. It is $\overline{\theta^2} = 24\ kTt/\pi\eta l^3$, where k is the Boltzmann constant, T is the absolute temperature, η is the coefficient of viscosity of the medium, l is the length of the cylinder, and t is the time interval between observations of the angle θ through which the sphere rotates. It must be understood that $\overline{\theta^2}$ represents the resultant mean-square angular displacement due to a very large number of nudges from impacts of the substrate molecules. In all observations on rotational Brownian motion, however, the projection of the angle on a fixed plane is measured, so that the foregoing equation should be divided by 3 in applying it to experimental data.

Consider a rod-like bacterium 1.5 μ long and 0.5 μ in diameter in a medium at a temperature of 27°. Set η equal to 1 cpoise, t to 1 sec and k to $1.38 \cdot 10^{-16}$ erg/°. Then the ratio of the mean-square angle of rotation about a transverse axis to the time interval for observations equals $24 \times 1.38 \times 10^{-16} \times 300 \times 0.01 \times (1.5)^3 \times 10^{-12}$ or 9.3 radians^2 sec. On the average, therefore, the bacterium would rotate through approximately 175°, almost a complete about face, during a 1-sec time interval. Thus locomotion in a given direction long enough to measure the speed, or even to allow the bacterium to go anywhere, is practically impossible unless provision is made to reduce the effects of rotational Brownian motion. A flagellum or flagellar tress several tens of μ long would prevent undue rotations; in effect, it would act as a stabilizer for the bacterium. If the over-all length is increased by a factor of 10 the root-mean-square angle is reduced by a factor of 1/30. REICHERT's observations that, by and large, small bacteria have longer flagella than large bacteria[10] support this hypothesis.

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[1] G. KNAYSI, *Elements of Bacterial Cytology*, 2nd ed., Ithaca, Comstock, 1951.
[2] M. P. STARR AND R. C. WILLIAMS, *J. Bacteriol.*, 63 (1952) 701.
[3] L. W. LABAW AND V. M. MOSLEY, *Biochim. Biophys. Acta*, 16 (1954) 325; 17 (1955) 322.
[4] I. W. SMITH, *Biochim. Biophys. Acta*, 15 (1954) 20.
[5] A. L. HOUWINK AND W. VAN ITERSON, *Biochim. Biophys. Acta*, 5 (1950) 10.
[6] C. C. BRINTON, A. BUZZELL AND M. A. LAUFFER, *Biochim. Biophys. Acta*, 15 (1954) 533.
[7] A. PIJPER, *J. Pathol. Bacteriol.*, 47 (1938) 1; 58 (1946) 325.
[8] A. EINSTEIN, *Ann. Phys.*, 17 (1905) 549; 19 (1906) 371.
[9] J. B. PERRIN, *Atoms*, translated by D. L. HAMMICK, Constable, London, 1920.
[10] K. REICHERT, *Zentr. Bakteriol. Parasitenk.*, I Origi., 51 (1909) 14.
[11] K. E. MACHIN, *J. Experimental Biol.*, 35 (1958) 796.

Received March 28th, 1960

Biochim. Biophys. Acta, 42 (1960) 344–345

Masked condition of ionic residues in collagen

Physico-chemical studies on collagen have emphasized the importance of hydrogen bonds in the structure. However, the state of the ionized residues in this protein is still obscure, although there is evidence from electron microscopy that they may have a special orientation in the fibril[1]. Also, analysis of titration curves reveals unusual behavior in that there is slight reactivity over the range pH 6–10, and a low alkali-neutralizing capacity compared to the content of basic amino acids[2]. This and other evidence mentioned by GUSTAVSON suggests the existence of salt-like crosslinks in collagen[3]. Recent observations dealing with the interaction of collagenous materials with salt are those of VEIS *et al.*[4], who found that acid-precursor gelatin bound about 200 μmoles NaCl/g from 0.1 M NaCl solution. This corresponds to about 20% of the protein's capacity, judged from the content of ionic residues. At neutral reaction, ARMSTRONG[5] detected no binding of K^+ by insoluble collagen, but noted a binding of about 65 μmoles/g of acid from solutions of low ionic strength.

The distribution of NaCl between collagen* and external salt solutions was examined in order to obtain additional information on the reactivity of the ionic residues. In these experiments, 50–100 mm³ pieces of collagen were equilibrated with various concentrations of NaCl (1–2 l) for several days at 3°. Next, they were removed and blotted with paper tissues or pressed mechanically, weighed, dried *in vacuo* over P_2O_5, and reweighed to obtain the water content and dry weight. The dry protein was extracted several times with 2-ml portions of 0.1 N HNO_3 (see ref. 6), and sodium and chloride were determined in the combined acid extracts and in the medium.

When insoluble collagen is equilibrated with a 1000-fold excess of 0.15 M NaCl, it is reasonable to assume that Na^+ and Cl^- are the only mobile ions present in significant amount in the protein phase. Since the concentrations of both these ions in the collagen phase have been determined, the net charge, which is available for interaction with salt, can be obtained by application of the condition of electroneutrality. This may be represented as

$$(X^+) = (Cl^-)_c - (Na^+)_c,$$

where (X^+) is the net charge and $(Cl^-)c$ and $(Na^+)c$ are the concentrations of Na^+ and Cl^- ions in the protein phase. The magnitude of this available charge, (X^+), should be independent of the nature of the interaction of the salt with the macromolecules.

If the protein is compressed mechanically after equilibration with a salt solution, Na^+ and Cl^- ions will be removed in equivalent amount, and the net charge should remain unaltered. The results of such an experiment are presented in Table I. The protein was subjected to hydraulic forces while sandwiched between cotton cloths and cellulose tissues in order to remove entrained solution, and analyzed for the mobile ions remaining. The original water content of 54% decreased to 26% at an applied force of 6–10 tons. The chloride concentration in the collagen phase increased (Column 2) as solution was expressed by the force applied, illustrating that there was interaction of chloride with cationic groups of the protein. On the other hand, the sodium concentration in the collagen phase decreased significantly as solution was removed from the protein, suggesting that there was no interaction of sodium and protein ions. This is concluded because, if there were appreciable interaction, the concentration of Na^+ remaining in the collagen phase should increase as progressive removal of water increases the concentration of immobile charged anionic groups. The amount of sodium found in the collagen subjected to a force of 6 tons or greater was only 25 μmoles/g. Table I shows that the value of X, the content of available cationic sites, was remarkably constant under the experimental conditions. Since no interaction with Na^+ was detected, there appear to be negligibly few available free anionic sites compared to the number of available cationic sites in collagen.

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* Steerhide collagen was generously supplied by Dr. F. O'FLAHERTY of the University of Cincinnati. It was prepared by extraction with 8% NaCl and dehydration with alcohol and acetone.

TABLE I

CONSTANCY OF AVAILABLE CHARGE OF COLLAGEN AS CONTENT OF ENTRAINED SOLUTION IS VARIED AFTER EQUILIBRATION WITH 0.15 *M* NaCl

Force *tons*	$(Cl^-)_c$* *mM*	$(Na^+)_c$* *mM*	*Available charge (X)**,** *µmoles/g dry wt.*
0	201 ± 2	100 ± 3	118 ± 3
0.5	248 ± 3	96 ± 9	104 ± 7
1	270 ± 3	80 ± 7	107 ± 2
2	301 ± 7	73 ± 6	106 ± 1
4	331 ± 6	70 ± 8	105 ± 2
6	367 ± 5	70 ± 5	109 ± 4
8	373 ± 6	68 ± 1	109 ± 1
10	364 ± 6	67 ± 1	108 ± 3
		Average	108 ± 3

* Mean values for triplicate samples ± average deviation.
** Available charge on collagen obtained from

$$X = ((Cl^-)_c - (Na^+)_c)\ (\text{G entrained } H_2O)/\text{dry wt.}$$

The influence of the concentration of NaCl on the nature of the collagen–NaCl interaction was also studied. The media had final pH 5.0–6.0, and the data are summarized in Table II. It is evident that there was always a marked excess of chloride and deficit of sodium in the collagen phase compared to the medium phase. Again, this is the expected result if the protein has a net positive charge. The magnitude of the charge shown in column 4 agreed well with the values obtained in 0.15 *M* NaCl except when the NaCl concentration was less than 19.5 mM. The last column in Table II gives the excess of chloride associated with the collagen over and above that predicted by the Donnan relationship. This excess decreased with increasing concentrations of NaCl in the medium. No adequate explanation for this can be offered at the present time. However, it does not seem to be an osmotic effect on the

TABLE II

DISTRIBUTION OF NaCl BETWEEN COLLAGEN AND MEDIUM

Concentration (mM)			*µmoles/g dry weight**	
	Collagen phase			
Medium phase $(Na^+)_m$, $(Cl^-)_m$	$(Cl^-)_c$	$(Na^+)_c$	*Net charge (X)***	*Excess chloride (E)****
2.5	51	0.6	79 ± 1.3	65 ± 4.8
9.8	64	3.1	94 ± 3.0	51 ± 4.1
19.5	79	7.7	107 ± 2.4	43 ± 3.4
49	102	29	106 ± 1.3	27 ± 2.7
97	147	67	110 ± 2.0	10 ± 5.4

* Mean values for 6 samples ± standard deviation.
** Method of computation of X given in notes under Table I.
*** Excess of chloride in collagen phase obtained from

$$E = ((Cl^-)_c^{\text{Observed}} - (Cl^-)_c^{\text{Donnan}})\ (\text{G entrained } H_2O)/\text{dry weight}$$

where

$$(Cl^-)_c^{\text{Donnan}} = (Na^+)_m\ (Cl^-)_m/(Na^+)_c.$$

collagen structure since the water content of the protein decreased only from 61% in 2.5 mM NaCl to 58% in 97 mM NaCl. To summarize, the data in Table II show that, even at salt concentrations in which the system deviated appreciably from an ideal Donnan distribution, the net charge on the collagen remained constant at the level found in 0.15 M NaCl, where a simple Donnan relationship prevailed.

These results may be considered in connection with the amino acid composition of native bovine collagen. The excess of basic over acidic groups is about 120 µmoles/g[7,8]. It may be noted that the available charge found in this study is close to this value. Therefore, the data suggest that only the excess basic residues in collagen are available for interaction with NaCl. Since the sum of the free basic and acidic protein groups is about 1700 µmoles/g[7], it is concluded that approximately 95% of the ionic sites in steerhide collagen most probably exist in some sort of internal compensation.

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[1] R. S. BEAR, *Advances in Protein Chem.*, 7 (1952) 69.
[2] J. H. BOWES AND R. H. KENTEN, *Biochem. J.*, 43 (1948) 358.
[3] K. H. GUSTAVSON, *The Chemistry and Reactivity of Collagen*, Academic Press, New York, 1956,
[4] A. VEIS, J. ANESEY AND J. COHEN, in G. STAINSBY, *Recent Advances in Gelatin and Glue Research.* Pergamon Press, New York, London, Los Angeles, Paris, 1958, p. 155.
[5] D. M. G. ARMSTRONG, in G. STAINSBY, *Recent Advances in Gelatin and Glue Research*, Pergamon Press, Oxford, 1958, p. 262.
[6] H. L. KERN, *A.M.A. Arch. Ophthalmol.*, 52 (1954) 131.
[7] J. E. EASTOE, A. A. LEACH, in G. STAINSBY, *Recent Advances in Gelatin and Glue Research*, Pergamon Press, New York, London, Los Angeles, Paris, 1958, p. 173.
[8] J. H. BOWES, R. G. ELLIOTT and J. A. MOSS, *Biochem. J.*, 61 (1955) 143.

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Dialysis of imidazole compounds from frog-muscle suspensions*

It has been suggested that in striated muscle, carnosine forms part of a more complex, biochemically active compound[1]. This hypothesis was prompted by the failure of many workers to recognize a function attributable to carnosine[1–3] and it has been supported by reports that carnosine phosphates exhibit pronounced effects *in vitro*[4,5]. However, no carnosine-containing compound has been isolated from muscle extracts, whereas free carnosine has been obtained repeatedly from the muscle of several species. The isolation methods have required a substantial period of time and, in many cases, considerable chemical manipulations. Thus, it remains possible that complex carnosine compounds have been decomposed during experimental manipulation.

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This report describes a study of the diffusion of the imidazole-containing compounds of muscle through cellophane membranes. Frog muscle was used for several reasons. Frog muscle-nerve preparations are known to function over prolonged periods at room temperature, and homogenates of frog muscle carry on oxidative reactions for several hours. The muscle contains high levels of carnosine so that a small amount of suspension provides sufficient compound for study. The muscle can be worked up rapidly and frogs respond satisfactorily to drugs which are known to affect the state of striated muscle. Most importantly, cold-blooded animals can be chilled before sacrifice, reducing post-mortem changes to a minimum.

Dialyses were conducted with an apparatus similar to that of CRAIG *et al.*[6] but modified as described elsewhere[7]. With the CRAIG apparatus, a small volume of sample was dialyzed through Visking dialysis tubing with large surface area and an average diffusion distance of about 1 mm. Duplicate dialyses with different commercial cellophane membranes were conducted on each sample.

Rana pipiens, females, weighing about 70 g were used. Group 1 consisted of four normal animals. Group 2 was made up of three frogs anesthetized with 4 or 8 ml of 25 % $MgSO_4$ injected intraperitoneally 10 min before sacrifice. The muscle from these animals lacked tone and was noticeably soft. Group 3 contained three frogs injected subcutaneously with 2 units of curare (intocostrin, Squibb) in 0.1 ml water. The animals were paralyzed in 20–30 min at which time they were killed. Group 4 consisted of three frogs which were injected intraperitoneally with 1 ml 2 % NaF 20 min before they were sacrificed. At that time the frogs were sluggish but showed no muscular activity or other gross effects of the treatment.

The frogs were brought to 3° at least 15 min before being beheaded, and the muscles from both thighs were pooled for immediate use. The tissue was minced and homogenized with an equal weight of isotonic KCl. The suspension was strained through muslin and a portion was introduced into the sample chamber. All work was conducted in the cold room with equipment pre-cooled to 3°. The dialyses were started 15–20 min after beheading of the animals.

In preliminary experiments, it was found that the relative rate of dialysis of pure carnosine in solution was independent of moderate changes in the volume of sample, the concentration of carnosine, pH (between 6.6 and 7.3), the ionic strength of the bath, and the presence of human serum albumin. Increasing the temperature from 3° to 25° caused a 5-fold increase in the rate of dialysis. Although the rate was not defined by a simple exponential equation, deviation from the theoretical pattern was reproducible, appearing to be a function of the apparatus. Other tests demonstrated that all of the Pauly-positive imidazole compounds of the muscle were free to diffuse into the dialysate.

There is no significant difference between any of the rates for suspensions and those for pure carnosine (Table I). This is especially significant in these studies because of the limited possibility for degradation of the muscle compounds. Approx. 60 % of the "carnosine" diffused from the muscle proteins during 20 min of dialysis, *i.e.* within 40 min of the beheading of the animal.

The data do not support the suggestion that muscle carnosine exists as part of a larger molecule. The results do not exclude this hypothesis, but do impose limitations upon the nature of such a complex compound should it exist. There are three situations in which our data would be consistent with the existence of such a compound.

TABLE I

RATE OF DIALYSIS OF CARNOSINE AND FRESH FROG MUSCLE IMIDAZOLE COMPOUNDS

Source fo sample	*Average amount(as %) of dialyzable imidazole remaining in dialysis bag after*			
	3 min	*8 min*	*20 min*	*80 min*
Carnosine	75	55	37	17
Normal frogs	78	57	38	19
$MgSO_4$-treated frogs	74	54	36	15
Curare-treated frogs	75	53	35	17
NaF-treated frogs	77	56	38	17

1. The presence of a "Pauly positive" compound behaving differently from carnosine might not have been detected if its concentration represented less than 10% of the total diffusible muscle imidazole.

2. A carnosine compound which decomposed within 20 min at physiological pH and at 3°, *i.e.* before the dialysis was started, would not be detected. Attempts to modify the muscle to prevent such decomposition, if it occurred, were not successful. One type of easily hydrolyzable compound meeting this criterion would be the reversible binding product of metal ions and carnosine. In other experiments[7], we have compared the titration curves of carnosine in the presence of several metal ions. No evidence was obtained for a significant degree of chelation between carnosine and Ca^{++}, Mg^{++}, Li^{+}, K^{+} or Rb^{+}.

3. A complex compound with a diffusion rate the same as that of carnosine would not have been detected in the dialysis experiments. CRAIG AND KING[8] have demonstrated that, in their system, molecular size is the chief factor governing dialysis rates. Thus, the hypothetical compound would have to be of the same general size as carnosine (mol. wt. 226). Further, it would have to be labile to account for the ready isolation of carnosine from muscle.

Our studies are of further interest in view of a report by EGGLETON AND EGGLETON[9], who obtained data suggesting that carnosine cannot move through the membrane of intact muscle cells. In contrast to carnosine, histidine was freely diffusible, even though its size and the p*K*'s of its functional groups are similar to those of carnosine. Since carnosine diffuses rapidly through cellophane membranes, its failure to pass through muscle membranes cannot be due to spontaneous formation of non-diffusible, polymolecular aggregates. Further studies of cell permeability to imidazoles are needed to clarify the status of carnosine in living muscle.

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[1] V. DU VIGNEAUD AND O. BEHRENS, *Ergeb. Physiol. biol. Chem. u. exptl. Pharmakol.*, 41 (1939) 917.
[2] D. C. SUTFIN AND H. M. HINES, *Am. J. Physiol.*, 186 (1956) 286.
[3] C. J. PARKER, JR. AND W. D. LANGLEY, *Biochim, Biophys. Acta*, 37 (1960) 537.
[4] S. E. SEVERIN, E. F. GEORGIEVSKAYA AND V. I. IVANOV, *Biokhimiya*, 12 (1947) 35.

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[5] M. C. Goodall, *Nature*, 178 (1956) 539.
[6] L. C. Craig, T. P. King and A. Stracher, *J. Am. Chem. Soc.*, 79 (1957) 3729.
[7] F. G. Bock, *Univ. Microfilms*, L. C. Card No. Mic 58-2889 (1958).
[8] L. C. Craig and T. P. King, *J. Am. Chem. Soc.*, 77 (1955) 6620.
[9] M. G. Eggleton and P. Eggleton, *Quart. J. Exptl. Physiol.*, 23 (1933) 391.

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The uptake of [^{3}H]uridine in normal and filamentous forms of *Escherichia coli* infected with T-2 bacteriophage

Hershey[1] and Volkin and Astrachan[2] have shown that although net RNA synthesis almost comes to a complete halt during T-2 phage infection, a small amount of ^{32}P becomes incorporated into RNA nucleotides, indicating the occurrence of some RNA turnover. In the course of investigating sites of RNA synthesis in *E. coli* the existence of a concurrent uptake of uridine was established. Some features of this uptake will be described here.

We first considered the uptake of [^{3}H]uridine (obtained from New England Nuclear Corp.) by a culture of *E. coli* B in minimal A-1 medium[3], aerated at 37°, and infected with approximately 20 T-2 bacteriophages per cell. [^{3}H]uridine, at a concentration of 50 μC/ml (specific activity, 680 mC/mmole) was added to the culture 3 min after infection. The final cell concentration was $3.5 \cdot 10^7$ cells/ml. Colony counts following 10^4 dilution of the aliquots of this culture revealed that at this point less than 1 % of the cells were non-infected. Samples (5 μl in volume) from the culture were taken at regular intervals and placed on stainless-steel planchets in a 10 % aq. solution of formalin containing a high concentration of unlabeled uridine to reduce adsorption of label to the metal surface. The cells were fixed for 10 min, dried, fixed again for 10 min in Carnoy solution, transferred to distilled water through graded alcohols, and dried again. Counts were made in a windowless flow Geiger counter. A correction was made for background due to adsorption of label to the planchet and other causes.

Fig. 1 shows that the uridine content of the cells rises at a linear rate for about 10 min, then reaches a plateau for the next 20 min. The initial rate of uptake is much higher than that expected for the non-infected cells present at that time. It must be concluded therefore that this uptake takes place in the infected cells. It has been shown that in normal cells 90 to 95 % of the incorporated [^{3}H]uridine appears as RNA uridine and cytidine, the rest appearing as DNA deoxycytidine[4]. Treatment with RNAase of the phage-infected cells removed approx. 90 % of the label. It is, therefore, inferred that [^{3}H]uridine uptake is an indication of RNA synthesis or turnover. One difference should be noted between our results and those of Volkin and Astrachan[2]. They found that in minimal medium phosphorus turnover in RNA continues throughout the entire latent period, while in our experiment the

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

[^{3}H]uridine uptake seems to stop after about 10 min (behavior corresponding to the turnover of phosphorus in peptone broth). Whether this reflects differences in experimental conditions or a basic difference between the uptakes of uridine and phosphorus has not been ascertained here. Experiments conducted on *E. coli* B in ANDERSON's M-9 medium or in nutrient broth and on filamentous forms of *E. coli* B gave results similar to those expressed in Fig. 1, but with greatly varying initial rates of uptake and final amounts of incorporation.

The uptake of [^{3}H]uridine by T-2 bacteriophage-infected normal cells and filaments was also studied by radioautography. In one experiment *E. coli* B filaments (obtained by irradiation with a ultraviolet dose of 20 ergs/mm^2 at 265 mμ), averaging about 15 μ in length, and growing exponentially, were infected with approx. 50 T-2 bacteriophages per cell and, after 5 min, inoculated with 12 μC/ml [^{3}H]uridine. 6 min later the cells were placed on a prepared glass slide on a drop of 10 % formalin in water containing a high concentration of unlabeled uridine, fixed for 10 min, dried, placed in osmium tetroxide vapors for 5 min, washed 3 times in distilled water and dried. They were radioautographed, using the stripping-film technique[5], with Kodak Ltd. AR-10 emulsion. After suitable exposure, and development of the slides, they were examined in a phase-contrast microscope, the cell length measured by means of a micrometer disc, and the number of photographic grains associated with each cell counted in bright field. This number of grains was assumed to be proportional to the [^{3}H]uridine uptake of the cell[6]. Fig. 2 presents the results of this experiment. It can be seen that the uptake of uridine in T-2-infected filaments is directly proportional to length.

This proportionality could be linked to either the number of bacteriophages

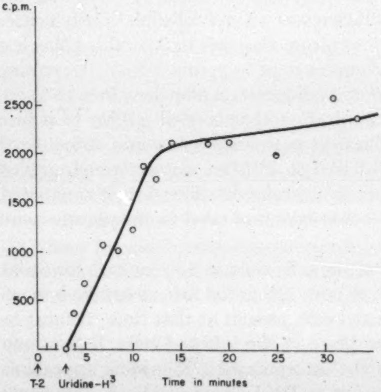

Fig. 1. Uptake of [^{3}H]uridine by *E. coli* B infected with T-2 bacteriophage. The [^{3}H]uridine was added 3 min after inoculation with 20 bacteriophages/cell.

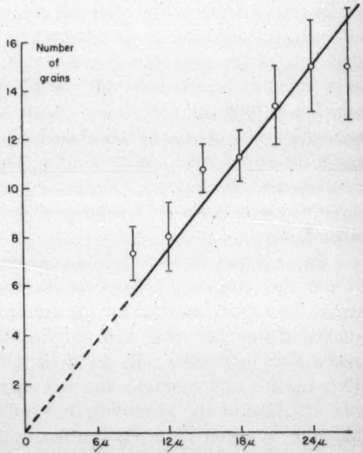

Fig. 2. Uptake of [^{3}H]uridine by filamentous forms of *E. coli* B infected with T-2 bacteriophage, *versus* cell length. Each point indicates the average number of radioautographic grain counts for cells of a given length class. A few non-infected cells present in the preparation were not included in calculating the averages.

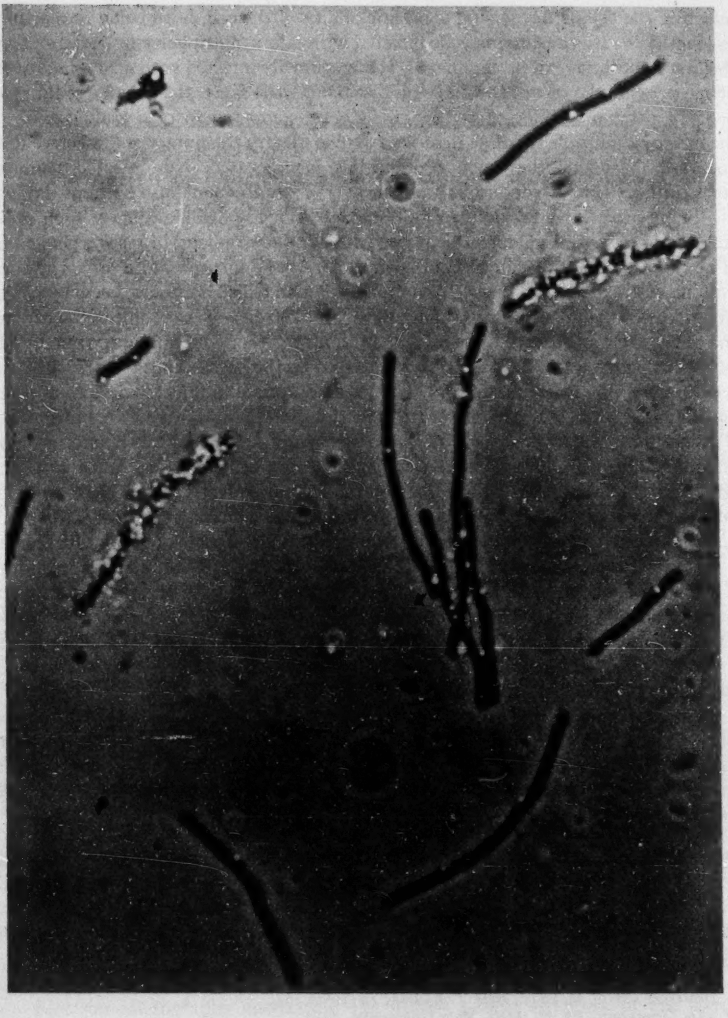

Fig. 3. Radioautograph of *E. coli* B filaments grown in the presence of [^{3}H]uridine following infection with one T-2 bacteriophage per cell. The photographic grains, seen slightly out of focus in phase contrast, appear as white spots. Two non-infected cells show a high uptake of [^{3}H]uridine. In the infected cells the grains appear in random places along the length of the filament.

infecting the cell or the general synthetic ability of the cell, both being proportional to length[3,4,7]. By considering cells infected with a low multiplicity of bacteriophages the first hypothesis can be eliminated. If filaments are infected with an average of one phage per cell, the rest of the procedure remaining the same, the average grain count per day per 6 μ of infected cell length is 0.41, compared to 0.39 grain/day/6 μ in the case of a multiplicity of 50 described above. Clearly the number of infective units does not determine the amount of uridine incorporated. Non-infected filaments labeled under similar conditions gave an average grain count of 6.8 grains/day/6 μ.

Some qualitative results can be obtained from the examination of such radioautographs (Fig. 3). In filaments infected with a low multiplicity of T-2 the percentage of non-infected cells (easily recognized by their heavy incorporation of [^{3}H]uridine) was, in one experiment, 24 %. Assuming a Poisson distribution of phage particles among cells it might be inferred that about 35 % of the cells were infected with only one phage. It was observed in this preparation that the reduction in uridine uptake related to phage infection was always extended to the entire length of the filament, even in very long cells (up to 30 μ). The reduced uptake in infected cells took place similarly, along the entire length of the cell. Thus, in regard to the two manifestations of phage infection studied here, decrease in uridine uptake and distribution of the small amount incorporated, even very long filaments behave as one single unit. This agrees with our findings on uridine uptake by non-infected filaments[4], and also with the results of DEERING[8], who reached a similar conclusion with regard to still another property of filaments (colony formation) from the results of radiation studies.

In conclusion, we found that T-2 phage-infected *E. coli* B and filamentous forms of this strain incorporate [^{3}H]uridine for a short period of time at a reduced rate and that the amount of label incorporated is proportional to the length of the cell but not to the multiplicity of infection. The reduction in uptake affects the entire length of the cell, even in long filaments, but similarly the uptake still present is not localized even when the cell is infected with one bacteriophage.

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[1] A. D. HERSHEY, *J. Gen. Physiol.*, 37 (1953) 1.
[2] E. VOLKIN AND L. ASTRACHAN, *Virology*, 2 (1956) 149.
[3] R. A. DEERING AND E. CHRISTIAN, *Virology*, 6 (1958) 150.
[4] L. G. CARO, *Ph. D. thesis*, Yale University, 1959.
[5] I. DONIACH AND S. R. PELC, *Brit. J. Radiol.*, 23 (1950) 184.
[6] R. VAN TUBERGEN, *Ph. D. thesis*, Yale University, 1959.
[7] R. A. DEERING, *J. Bacteriol.*, 76 (1958) 123.
[8] R. A. DEERING, *Biochim. Biophys. Acta*, 31 (1959) 11.

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Preliminary Notes

Formation of helical strands by sodium deoxycholate as revealed by electron microscopy

In experiments in which vaccinia virus was exposed to 0.5- 1.0 % sodium deoxycholate at pH 7, and then dialyzed for 2–3 h against distilled water, subsequent electron micrographs revealed long fibers surrounding the few virus particles which survive such treatment. These fibers were not seen when the virus–deoxycholate mixture was dialyzed for 10–15 h, or when virus was inactivated by urea or guanidine-hydrochloride[1].

Typical electron micrographs of purified vaccinia virus treated with 0.9 % sodium deoxycholate and dialyzed for 2–3 h are shown in Figs. 1a and 1b. Samples of the dialyzed suspensions were mounted directly on Formvar-coated grids, air-dried in the presence of osmium vapor for 1–2 h, shadowed with chromium, and examined in a RCA-3B electron microscope. The majority of virus particles surviving deoxycholate treatment appear grossly normal, as will be described in detail elsewhere. It will be seen, however, that the virus particles are surrounded by masses of fibers of indefinite length and of remarkably uniform helical structure (Figs. 1a, 1b). The smallest fiber diameter is approximately 40 Å (Fig. 1b); larger fibers (diameter approximately 100 Å) frequently appear in parallel sheets (Fig. 1a). These latter fibers are possibly composed of two or more strands of 40 Å diameter fibers, and in other micrographs two fine strands were seen "unwinding" from the ends of thicker helical fibers.

The addition of deoxyribonuclease together with 0.005 *M* $MgCl_2$ to the virus-deoxycholate suspension caused a flocculent precipitate which carried down most of the fibers. It was then observed that deoxycholate alone formed a similar precipitate on the addition of traces of $MgCl_2$. Solutions of sodium deoxycholate (0.5 % in distilled water) were therefore placed directly on grids, exposed to osmium vapor, shadowed with chromium, and examined in the usual way. Long helically-wound fibers of similar dimensions were again seen (Fig. 1c). Identical structures were obtained from two samples of commercially purified deoxycholate*. In general, the detailed helical structure was most clearly revealed in preparations that also contained virus, and micrographs of deoxycholate alone seldom showed the helices as clearly as in Fig. 1c.

Electron micrographs illustrating the fibrous helical structure of the steroid have not been previously reported in the literature. RICH AND BLOW[2] carried out X-ray diffraction studies on fibers drawn from viscous complexes of sodium deoxycholate and glycylglycine, and concluded that the complex has the form of a helix; the *a* axis of the helix varied from 36.2–49.1 Å, depending on the mole ratio of glycylglycine to deoxycholate. Further detailed studies by these authors on deoxy-

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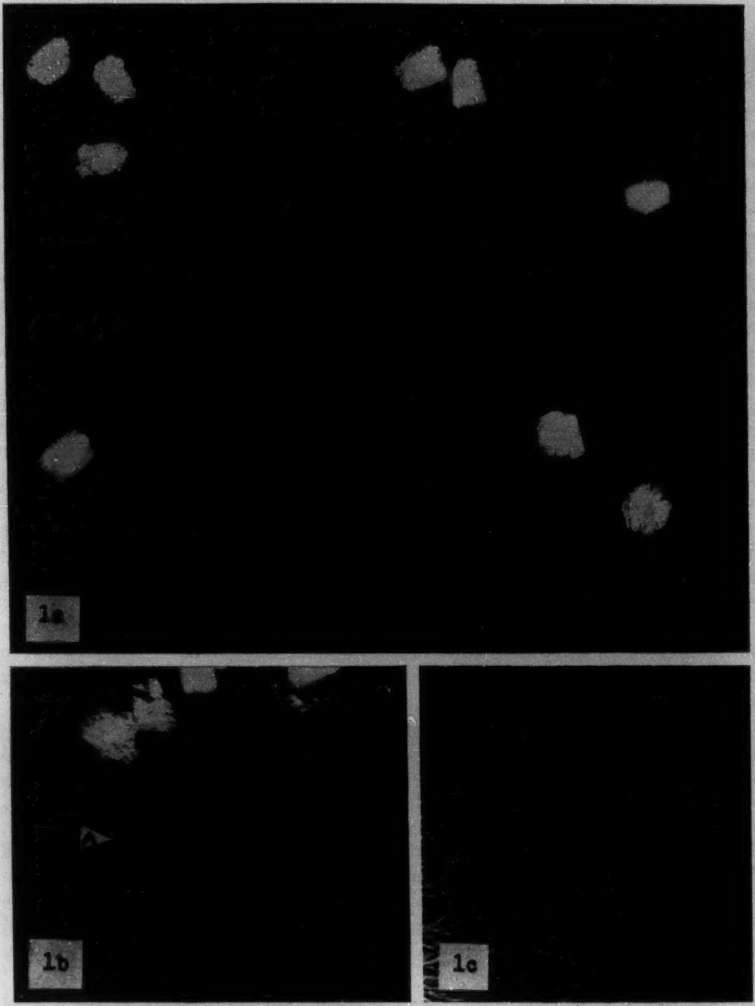

Fig. 1. Electron micrographs of vaccinia virus-deoxycholate mixtures and of pure deoxycholate. (a) Typical appearance of virus-deoxycholate suspension after dialysis for 3 h. (b) Similar preparation showing a fine fiber (A). (c) Sodium deoxycholate alone.

cholate and other steroids have shown that gel formation is determined by various factors including pH, ionic strength, and type of ion in the buffer[3]. The diameter of the smaller fibers seen in our electron micrographs is closely similar to that calculated from X-ray diffraction patterns[2], and the helical structure predicted from X-ray diffraction is strikingly revealed in the electron microscope.

Deoxycholate has been widely used to prepare deoxyribonucleic acid from bacteria[4] and infectious "ribonucleic acid" from animal viruses[5]. Since the steroid may form long helical fibers under certain conditions, and hence dialyzes slowly, electron micrographs taken after using it should be interpreted cautiously.

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[1] J. F. MCCREA, S. ANGERER, AND J. O'LOUGHLIN, *Bacteriol. Proc.*, (1960) 125–126.
[2] A. RICH AND D. M. BLOW, *Nature*, 182 (1958) 423–426.
[3] D. M. BLOW AND A. RICH, *J. Am. Chem. Soc.*, in the press.
[4] R. D. HOTCHKISS, in S. P. COLOWICK AND N. A. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 692.
[5] S. G. ANDERSON AND G. L. ADA, *Virology*, 8 (1959) 270–271.

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Type of attachment of sialic acid in ox-brain mucolipid

Sialic acid is easily liberated from brain mucolipid preparations under mildly acid conditions[1,2]; it may thus partly be attached terminally to the rest of the glycolipid polymer in a comparatively labile glycosidic linkage. Since structural models proposed heretofore for these interesting and complex substances are entirely hypothetical, we have undertaken a study of the various linkage types supporting the architecture of the polymer. We limit ourselves here to a consideration of sialic acid and to the behavior of a purified ox-brain mucolipid, both intact and after hydrolytic removal of sialic acid, towards oxidation by periodate (Fig. 1).

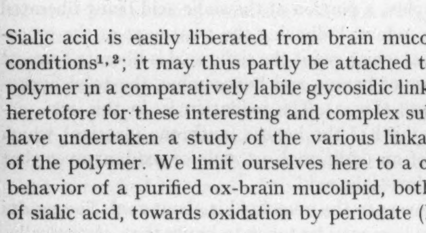

Fig. 1. Oxidation with metaperiodate at pH 4. I, free sialic acid; II, mucolipid after treatment with 0.1 N H_2SO_4 at 80° for 2 h; III, intact mucolipid.

The preparation of the mucolipid and, from it, of crystalline ovine sialic acid (5-N-acetylneuraminic acid) were described previously[2]. The reactions were carried out in 5 mM aqueous solutions of sodium metaperiodate around pH 4. Oxidant consumption was measured by titration of residual periodate to iodate at pH 7 with thiosulfate.

The theoretical consumption by one mole of the free sialic acid isolated from the mucolipid is 2 moles of periodate. Actually, there is some overoxidation (Fig. 1), 2.7 moles being consumed, most likely owing to the active methylene group in position 3[3, 4]. The intact mucolipid, containing 26 % sialic acid (w/w), reduced 1.7 mole of periodate per mole of polymer-bound sialic acid, whereupon its sialic acid content (by direct Ehrlich reaction[5]) dropped to one half. Only one half of the sialic acid molecules would hence appear to be strictly terminal, *i.e.*, bound only glycosidically at carbon-2, whereas the other half must be also substituted at least at the 8 hydroxyls, thus escaping destruction by periodate. The quantity of sialic acid attacked would have required the uptake of 1 or, if the explanation given above also holds for bound sialic acid, of 1.35 moles of oxidant. The small amount of additional periodate reduced is probably attributable to the cleavage of the few galactose units that, as shown in the following note[6], are terminal in the polymer.

The results are compatible with the provisional assumption that in the lipid polymer the bulk of the hexose molecules must be substituted, in addition to glycosidic attachment at carbon-1, either at position 3 or doubly at 2, 3 or 3, 4, and the N-acetyl hexosamine fraction similarly in position 3 or 4, in order to withstand attack by periodate. The following note[6] reports on methylation studies designed to clarify the situation.

Initial degradation of the mucolipid is not without influence on the subsequent course of oxidation. Brief pretreatment at pH 9 and room temperature augmented both the reaction rate and the extent of uptake of periodate. This effect on the polymer appears, however, to be complex, a portion of the sialic acid being liberated in form of a dialyzable degradation product. Following the treatment of the mucolipid with 0.1 N H_2SO_4 at 80° for 2 h, at which point the sialic acid is liberated almost completely without accompanying loss of hexose and hexosamine, the total hydrolysate consumed 3.2 moles of periodate (Fig. 1). The oxidation is, in this case, accompanied by the loss of about one third of the hexose (anthrone reaction) which must be responsible for the uptake of an additional 0.5 mole of oxidant over that reduced by an equivalent amount of free sialic acid.

The molar ratio of sialic acid to hexose in the mucolipid is about 2:3[2]. The results of the oxidation experiments described here may be taken to imply that, statistically, one out of three hexose moieties carries both sialic acid molecules, probably as a $2 \rightarrow 8$ linked dimer. The hexose molecule carrying the latter may, furthermore, be assumed to be doubly substituted along the 2, 3, and 4 positions, as the complete removal of sialic acid makes only a single vicinal diol of the hexose available for periodate attack. Experimental evidence points to galactose being the major sugar to which the sialic acid is attached.

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[1] A. ROSENBERG AND E. CHARGAFF, *Biochim. Biophys. Acta*, 21 (1956) 588.
[2] A. ROSENBERG AND E. CHARGAFF, *J. Biol. Chem.*, 232 (1958) 1031.
[3] D. B. SPRINSON AND E. CHARGAFF, *J. Biol. Chem.*, 164 (1946) 433.
[4] C. F. HUEBNER, S. R. AMES AND E. C. BUBL, *J. Am. Chem. Soc.*, 68 (1946) 1621.
[5] I. WERNER AND L. ODIN, *Acta Soc. Med. Upsaliensis*, 51 (1952) 130.
[6] J. D. KARKAS AND E. CHARGAFF, *Biochim. Biophys. Acta*, 42 (1960) 359.

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Methylation studies on ox-brain mucolipid

A large proportion (nearly 60 %) of the mucolipids consists of sugars (hexoses, hexosamine) and sialic acid[1]. In order to gain an insight into the backbone of these complicated structures it is, therefore, of interest to study the types of linkage supporting the various constituents. Preliminary information on the mode of attachment of sialic acid is presented in the preceding note[2]. Here, we discuss the results of methylation experiments designed to provide an understanding of the types of linkage involving the hexose moieties.

Purified mucolipid preparations from ox brain[1] were employed. For exhaustive methylation it was found convenient to use the methyl iodide–silver oxide arrangement with dimethylformamide[3] as the solvent. Methylation was repeated 6 to 8 times, with agitation of the mixture in the dark, but with decreasing quantities of the methylating reagent. The first treatment was performed in the cold (3°) for 40 h, the subsequent ones at room temperature, each time for 16 h. After each treatment the precipitate was washed with dimethylformamide and 4 times with chloroform; the combined extracts were, after dilution with more chloroform, clarified by filtration and concentrated to a small volume *in vacuo* at 35°. After every second or third methylation treatment the concentrated solution was first subjected to dialysis against running and distilled water (for 24 h each) and lyophilized.

The progress of the methylation was followed both by methoxyl determinations and by the infrared spectra (KBr plate) of the purified reaction products. The initially strong OH peak around 3600 cm^{-1} of the untreated mucolipid disappeared gradually until it reached the level of the control KBr plate. The spectroscopic test is more sensitive than the analytical one: even before the final stage the methoxyl content was already at a plateau of 25.3 %.

Measurements of osmotic pressure[4] indicated a molecular weight of 180,000 for the untreated mucolipid, in agreement with the value obtained previously by means of the ultracentrifuge[1], and of 200,000 for the fully methylated product. It is obvious that no appreciable degradation of the polymer had occurred during methylation.

The fully methylated mucolipid was methanolysed with 4 % methanolic HCl for 20 h at 105° in a sealed tube and then treated with 6 % aq. HCl for 100 min in a boiling-water bath. The solution was neutralized with Ag_2CO_3 and the filtrate, after being treated with H_2S and clarified, was passed through a short Amberlite IR-4B column and lyophilized. The residue, dissolved in little water, was examined chromatographically. First, separation on Whatman No. 1 filter paper was employed with several solvent systems and spraying reagents. *n*-Butanol-ethanol–water (4:1:5, v/v/v, top layer) and an aniline *p*-diodosalicylate spray reagent[1] were used routinely. After the preliminary identification of the spots by paper chromatography, a cellulose column and elution by ligroin–*n*-butanol–water (60:39:1, v/v/v) served for the separation of larger amounts of the methylated sugars for a more adequate identification.

Among the criteria of identification were the melting point, methoxyl content, infrared absorption, periodate oxidation and, when reference samples were available, chromatographic mobility. The following products of the hydrolytic cleavage of the methylated mucolipid thus were identified: (a) 2,3,4,6-tetra-O-methyl-D-galactopyranose; (b) 2,4,6-tri-O-methyl-D-galactopyranose; (c) 2,6-di-O-methyl-D-galactopyranose; and—but not yet fully identified—(d) 2,4,6-tri-O-methyl-D-glucopyranose.

The quantitative estimation of the methylated sugars utilized two methods employing separation by paper chromatography and colorimetry of the eluates. The compounds were estimated by means of the benzidine reaction[5] or of a reaction with aniline *p*-diiodosalicylate similar to a previously described procedure using aniline hydrogen phthalate[6]. It was found that (a) the recovery of the total hexoses of the intact mucolipid in form of their methyl derivatives was nearly quantitative; (b) the molar ratios of di- to tri- to tetramethylgalactose to trimethylglucose were 7:3:2:2. These figures represent the averages (corrected to the nearest integer) of seven separate determinations on three different preparations.

Before these observations as well as those outlined in the preceding note[2] can be applied unambiguously to a definition of the structure of the mucolipid molecule, more information will be needed concerning the other components of this polymer. We are continuing our efforts in this direction. A few general remarks may, however, be made provisionally. (1) The high proportion of dimethyl —and therefore triply linked— hexose indicates a high degree of branching. The low proportion, on the other hand, of tetramethyl hexose suggests that the side chains cannot consist only of hexose moieties. (2) Ether linkages between the sugar would have withstood the hydrolysis conditions employed. Since the existence of such linkages in the polymer seems unlikely, disaccharides not having been found in the hydrolysates, the only principal constituent capable of interconnecting the fatty acid–sphingosine–hexose units is sialic acid. To some extent, certain amino acids could also engage in cross-linking. (3) No simple repeating unit is apparent at present that would account for our observations.

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[1] A. ROSENBERG AND E. CHARGAFF, *J. Biol. Chem.*, 232 (1958) 1031.
[2] A. ROSENBERG AND E. CHARGAFF, *Biochim. Biophys. Acta*, 42 (1960) 357.
[3] R. KUHN, H. TRISCHMANN AND I. LÖW, *Angew. Chem.*, 67 (1955) 32.
[4] H. B. BULL AND B. T. CURRIE, *J. Am. Chem. Soc.*, 68 (1946) 742.
[5] J. K. N. JONES AND J. B. PRIDHAM, *Nature*, 172 (1953) 161.
[6] W. C. SCHAEFER AND J. W. VAN CLEVE, *Anal. Chem.*, 28 (1956) 1290.

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A ribonucleotide phosphorylase in chicken embryo

The ability of chicken-embryo slices or mince to incorporate certain nucleosides as enzymically formed nucleotides into the cellular DNA and the polynucleotide pyrimidines of RNA has been previously reported by FRIEDKIN *et al.*[1] and by REICHARD[2].

We have recently found a ribonucleotide-polymerizing enzyme in the homogenate from 9-day-old chicken embryos. The enzyme activity was found to be mainly in the material sedimented at 700 × *g*. The maximum activity of the crude enzyme, requiring the presence of Mg^{++}, was achieved at pH 7.2 but was still strong at pH 8.0, whereas at pH 9.4 no detectable activity could be observed. Since we performed our experiments with an unpurified fraction, no statement can be made as yet concerning the questions whether nucleotide diphosphates or triphosphates represent the only substrate for the polymerase, or whether there is need for a highly polymerized nucleotide strand as primer in the RNA-synthesizing process[3–6]. The presence of a nucleotide polymerase in embryonic extract, however, may help to elucidate its importance for cellular growth.

All experiments were performed in duplicate and measurements made against a control in which the enzyme fraction had been preheated at 100° for 3 min.

TABLE I

SPECIFIC ACTIVITIES OF THE SOLUBLE NUCLEOTIDE FRACTIONS (NORIT FRACTIONS)

The complete system contained in a total of 1.25 ml: 160.0 μmoles Tris–HCl buffer of appropriate pH, 2.0 μmoles $MgCl_2$, 0.2 μmole EDTA, 10.0 μmoles K^+, 5.0 μmoles ADP, AMP or ATP, 0.1 μmole KH_2PO_4, 20.0 μC ^{32}P ($3.1 \cdot 10^6$ counts/min), 0.25 ml corresponding to 660–725 μg protein of the homogenate or of the homogenate fraction in 0.25 *M* sucrose.

	Counts/min/μg P		
	pH 7.2	*pH 8.2*	*pH 9.5*
Complete system	1260	600	60
omit Mg	120	10	3
omit enzyme	0	0	0
omit ADP		negligible	
replace ADP by:			
ATP*	1050	265	8
AMP	18	10	2
Control	3	2	2

* The sample contained chromatographically detectable amounts of ADP.

After the reaction had been completed (60 min at 36.5°), the acid-insoluble material was precipitated by cold 0.3 *M* $HClO_4$. An aliquot of the supernatant after centrifugation—usually 0.1–0.2 ml—was adsorbed by partly deactivated Norit. Removal of the non-adsorbed material was accomplished by several washings with water initially containing traces of inorganic phosphorus. The adsorbed nucleotides were eluted with 50% ethanol containing NH_3 (pH 10.2). Table I shows the specific activity of the eluate at various pH's.

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid (dinatrium salt); ADP, adenosine diphosphate; AMP, adenylic acid; ATP, adenosine triphosphate; IMP, inosine monophosphate; IDP, inosine diphosphate.

In order to determine the localization of the polymerizing enzyme in the cell as well as that of the radioactivity of the compounds formed during enzymic action, a chromatographic separation of the Norit eluate from an experiment at pH 7.2 with ADP as substrate was performed with various homogenate fractions. After concentration *in vacuo*, the Norit eluates were applied to paper (Whatman No. 1) for chromatography in the butyric acid–NH_3 system. Spots were developed by u.v. light, cut out, and eluted with 0.02 *N* HCl. The respective adsorption data in the 250–290 mμ region as well as the phosphorus content were determined, and revealed the presence of AMP, ADP, ATP, IMP, and IDP. Correspondings samples of reference substances were run simultaneously.

TABLE II

LOCALIZATION OF POLYMERIZING ENZYME IN CHICKEN-EMBRYO CELL

	AMP	*ADP*	*ATP*	*IMP*	*IDP*
Whole homogenate	none*	2205	2630	—	
	50*	25	22**	120**	
Nuclear fraction	none	700	1640	negligible	—
(700 × *g* sediment)	170	220	155	20	traces
Mitochondrial fraction	none	60	430	negligible	—
(10,000 × *g* sediment)	145	215	90	not det.	—
Cytoplasmic fraction	none	45	80	—	60
(10,000 × *g* supernatant)	76	125	130**	98**	98**

* Top figure indicates specific activity (counts/min/μg P); bottom figure indicates mμmoles nucleotide present.

** Chromatographically not separated, calculated from adsorption data.

From the appearance of radioactive ATP in nearly equimolar amounts with inactive AMP (Table II), it can be concluded that a strong myokinase in addition to the nucleotide phosphorylase is present in the nuclear fraction, whereas in the cytoplasmic supernatant, where no polymerase activity is recorded, the deaminase action leading to inosine nucleotides becomes dominant.

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[1] M. FRIEDKIN, D. TILSON AND D. ROBERTS, *J. Biol. Chem.*, 220 (1956) 627.
[2] P. REICHARD, *Biochim. Biophys. Acta*, 27 (1958) 434; *J. Biol. Chem.*, 234 (1959) 1244.
[3] A. KORNBERG in R. ZIRKLE (ed.), *A Symposium on Molecular Biology*, Chicago, The University of Chicago Press, 1959, p. 31.
[4] S. OCHOA AND L. HEPPEL in W. D. MCELROY AND B. GLASS (eds.), *The Chemical Basis of Heredity*, Baltimore, Johns Hopkins Press, 1957, p. 615.
[5] S. OCHOA, *Federation Proc.*, 15 (1956) 832.
[6] L. EDMONDS AND R. ABRAMS, *J. Biol. Chem.*, 235 (1960) 1142.

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Studies on synthesis of soluble ribonucleic acid.
III. Analytical studies on soluble ribonucleic acid of rat liver

It has been shown[1,2] that the enzyme systems which incorporate ribonucleotide units into s-RNA can be fractionated from rat liver along with the s-RNA into 3 enzymically active ribonucleoproteins designated α, β, and γ. Each ribonucleoprotein can be fractionated further into the enzyme components and the s-RNA component[3]. The purpose of this report is to present analytical data on the s-RNA components fractionated from the parent ribonucleoproteins β and γ and on s-RNA prepared from the pH-5 enzyme fraction by extraction with phenol[4].

Analysis of alkaline hydrolysates of s-RNA β, s-RNA γ, and phenol-extracted s-RNA for their ribonucleoside content reveals the presence of adenosine, guanosine, cytidine, and uridine. From the quantities of these compounds given in the Table I,

TABLE I

COMPOSITION OF s-RNA β, s-RNA γ, AND s-RNA EXTRACTED FROM THE pH-5 ENZYME SYSTEM WITH PHENOL

10 mg each of s-RNA β and s-RNA γ isolated as described[3], and s-RNA extracted from the pH-5 enzyme fraction with phenol[4], all with $E_{260}:E_{230}$ ratios of 2.05–2.1, were hydrolysed in an excess of 0.1 N NaOH for 45 min at 85°. The hydrolysates were neutralized and then placed on Dowex-1 (200–400 mesh) formate columns (10 × 1 cm), and the elution was carried out by the method of COHN AND VOLKIN[10]. The ribonucleosides were eluted with 0.01 N HCOOH, concentrated to 10 ml *in vacuo*, and placed on Dowex-1 chloride columns (10 × 1 cm) in 0.01 M $K_2B_4O_7$. The elution was carried out according to the procedure described by COHN[11]. The u.v.-absorbing compounds in the fractions eluted from the columns were identified by comparing their positions in the chromatogram and their spectra in acid and alkali with those of authentic compounds. Pseudouridylic acid was identified by its position in the chromatogram and by the pronounced shift in the u.v. spectrum at 290 mμ between pH 11 and 12[12]. The compound listed as guanosine diphosphate has been identified, tentatively, as guanosine 3′(and 2′),5′-diphosphate on the basis of the following evidence: (1) The ratio guanine : ribose : phosphorus in the compound is 1.0 : 1.1 : 2.1; (2) the compound moves faster than guanosine 5′-diphosphate but slower than guanosine 5′-triphosphate upon electrophoresis on paper at pH 7.0; (3) under conditions known to result in the liberation of almost 1 mole of orthophosphate from 1 mole of guanosine 5′-diphosphate (10 min at 100° in 1.0 N HCl) no detectable orthophosphate is liberated from this compound.

	Ribonucleosides				
Component	*Adenosine*	*Uridine*	*Guanosine*	*Cytidine*	*Guanosine diphosphate**
s-RNA β	0.60	—	0.24	0.10	1.02
s-RNA γ	0.23	0.10	0.51	0.14	0.95
pH-5 s-RNA	0.67	0.12	0.14	0.07	1.21

	*2′,3′-Ribonucleotides**						
	AMP	*UMP*	*ψ-UMP*	*GMP*	*CMP*	*No of nucleotides per chain*	*Wt. of chain*
s-RNA β	7.2	6.0	1.01	13.0	13.2	42	15,000
s-RNA γ	6.0	5.0	1.21	11.1	11.0	36	13,800
pH-5 s-RNA	12.0	10.5	1.61	19.0	20.5	66	22,100

* Molar composition calculated by assuming that one terminal ribonucleotide residue corresponds to one polynucleotide chain.

Abbreviations: AMP, UMP, ψ-UMP, CMP, GMP, the mixed 2′-, 3′-isomers of adenylic, uridylic, pseudouridylic, cytidylic and guanylic acids, respectively; s-RNA, soluble ribonucleic acid.

it appears that most of the polynucleotide chains terminate with either adenylic acid or guanylic acid and that the proportion of chains which terminate in cytidylic acid is similar in all cases. The analysis of the alkaline hydrolysate also reveals the presence of guanosine 3′ (and 2′),5′-diphosphate in molar amounts equivalent to the sum of the ribonucleosides. This suggests that the ribonucleotide at the other end of all the chains is guanylic acid. Similar findings have been reported for s-RNA isolated from rabbit liver by a salt-extraction method[5] and for s-RNA isolated from *Escherichia coli* by a phenol-extraction method[6].

The determination of the average number of ribonucleotides per polynucleotide chain from the data in Table I gives values of 42 nucleotides for s-RNA β, 36 nucleotides for s-RNA γ, and 66 nucleotides for s-RNA extracted from the pH-5 enzyme fraction with phenol. These values correspond to chain weights of 15,000, 12,700, and 22,100 respectively. The chain weights of s-RNA β and γ are consistent with observed sedimentation coefficients ($S_{20,w}$) of 1.85 S[13].

Analysis of the hydrolysates for their ribonucleotide content shows that the ratio of pseudouridylic acid (ψ-UMP)[7,8] plus uridylic acid to adenylic acid and of cytidylic acid to guanylic acid is close to 1. Base ratios of this kind appear to be characteristic of the composition of s-RNA isolated from a wide variety of sources[5,6,9]. These findings are suggestive that base pairing may be of considerable importance in the structure and biological properties of s-RNA.

The results presented in this paper and studies on the physical characteristics of these s-RNA molecules will be discussed more fully in a later publication.

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[1] E. S. CANELLAKIS AND E. HERBERT, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 170.
[2] E. HERBERT AND E. S. CANELLAKIS, *Methods in Enzymology*, Vol. 6, Academic Press, New York, 1960, in the press.
[3] E. S. CANELLAKIS AND E. HERBERT, *Biochim. Biophys. Acta*, in the press.
[4] A. GIERER AND G. SCHRAMM, *Nature*, 177 (1956) 702.
[5] M. F. SINGER AND G. L. CANTONI, *Biochim. Biophys. Acta*, 39 (1960) 182.
[6] W. ZILLIG, D. SCHACHTSCHABEL AND W. KRONE, *Z. physiol. Chem.*, 318 (1960) 100.
[7] W. E. COHEN, *Biochim. Biophys. Acta*, 32 (1959) 569.
[8] F. F. DAVIS AND F. W. ALLEN, *J. Biol. Chem.*, 227 (1957) 907.
[9] G. W. CROSBIE, R. M. S. SMELLIE AND J. N. DAVIDSON, *Biochem. J.*, 54 (1954) 287.
[10] W. E. COHN AND E. VOLKIN, *Nature*, 167 (1951) 483.
[11] W. E. COHN, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 237.
[12] C. YU AND F. W. ALLEN, *Biochim. Biophys. Acta*, 32 (1959) 393.
[13] E. HERBERT AND E. S. CANELLAKIS, to be published.

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Distribution of adenylic acid residues in nucleic acid of tobacco mosaic virus

Recent studies on the nature of arrangement of purine and pyrimidine nucleotides in TMV–NA showed that they both occur "singly" and also in "clusters" of two or more[1–3]. Additional information on the mode of distribution of adenylic acid residues obtained by simultaneously digesting TMV–NA with two ribonucleases possessing different specificities, is presented in this communication.

The mechanism of degradation of RNA by pancreatic RNAase has been well established[4]. When RNA is digested with this RNAase the end products are cytidylic acid, uridylic acid and several oligonucleotides of varying lengths terminated by cytidylic acid and uridylic acid (equation I). Another RNAase differing in specificity from the pancreatic has been purified from takadiastase and its properties have been studied. Unlike pancreatic RNAase it specifically cleaves the secondary phosphate esters of guanosine 3′-phosphate[5]. We have recently purified this enzyme in our laboratory employing a modified procedure and used its specific action to study the distribution of guanylic acid residues in TMV–NA[3]. This enzyme will be hereinafter referred to as takaribonuclease-I (taka-RNAase-I). When RNA is digested with this enzyme I the end products are guanylic acid and several oligonucleotides of varying lengths terminated by guanylic acid (equation II). On the other hand if RNA is digested simultaneously with both the RNAase the expected end products are those given in equation III.

$$\text{RNA} \xrightarrow{\text{(pancreatic RNAase)}} \text{Cp} + \text{Up} + \text{ApCp} + (\text{Rp})_n\text{ApCp} + \text{ApUp} + (\text{Rp})_n\text{ApUp} \qquad \text{I}$$

$$\text{GpCp} + (\text{Rp})_n\text{GpCp} + \text{GpUp} + (\text{Rp})_n\text{GpUp}$$

$$(\text{Rp} = \text{adenylic acid, guanylic acid; } n = 1 \text{ or more})$$

$$\text{RNA} \xrightarrow{\text{(taka-RNAase)}} \text{Gp} + \text{ApGp} + (\text{Xp})_n\text{ApGp} + \text{CpGp} + (\text{Xp})_n\text{CpGp} + \text{UpGp} + (\text{Xp})_n\text{UpGp}$$

$$(\text{Xp} = \text{adenylic acid, cytidylic acid, uridylic acid; } n = 1 \text{ or more}) \qquad \text{II}$$

$$\text{RNA} \xrightarrow[\text{(taka-RNAase)}]{\text{(pancreatic RNAase)}} \text{Cp} + \text{Up} + (\text{Ap})_n\text{Cp} + (\text{Ap})_n\text{Up} + (\text{Ap})_n\text{Gp} \qquad \text{III}$$

$$(n = 1 \text{ or more})$$

It is evident from the products in equation III that it is possible to determine the extent to which the adenylic acid residues in RNA occur "singly" and in "clusters" by using the combined actions of the two RNAases. TMV–NA was digested exhaustively at pH 7.4 with both enzymes and the digestion products were separated by a two-dimensional technique involving electrophoresis and chromatography, details of which will be reported at a later date. The separated products were identified and estimated using the methods described earlier[1,2] and a summary of the results is given below.

Since the fragments containing adenylic acid in the TMV–NA digests were obtained as a result of the combined actions of pancreatic and taka-RNAases, which specifically cleave the secondary phosphate esters of pyrimidine ribonucleoside 3′-phosphates and guanosine 3′-phosphate, respectively, it can be assumed that

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Abbreviations: RNA, ribonucleic acid; TMV–NA, nucleic acid of tobacco mosaic virus; C, cytidine; A, adenosine; G, guanosine; U, uridine; p, phosphate.

these fragments occur in the intact TMV–NA preceded by any one of the three nucleotides, Cp, Up, and Gp. Of the total adenylic acid residues in TMV–NA 19.1 %, 19.6 % and 17.9 % occur "singly" as NpApCp, NpApUp and NpApGp, respectively; 12.1 %, 12.9 % and 12.8 % occur in "clusters" as NpAp(Ap)$_n$Cp, NpAp(Ap)$_n$Up and NpAp(Ap)$_n$Gp, respectively (where Np is is any one of the three nucleotides, Cp, Up, and Gp; $n = 1$ or more). The longest polyadenylic acid fragments so far isolated contained three adenylic acid residues. The possibility of the occurrence in TMV–NA of extremely small amounts of polyadenylic acid fragments containing more than three adenylic acid residues can not, however, be excluded.

Ful details of this investigation will be published elsewhere.

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[1] K. K. REDDI, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 293.
[2] K. K. REDDI, *Biochim. Biophys. Acta*, 36 (1959) 132.
[3] K. K. REDDI, *Nature*, (1960) in the press.
[4] L. A. HEPPEL AND J. C. RABINOWITZ, *Ann. Rev. Biochem.*, 27 (1958) 613.
[5] K. SATO AND F. EGAMI, *J. Biochem. (Japan)*, 44 (1957) 753.

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Radiosensitivity of nuclear RNA

Very little is known of the function of RNA in the cell nucleus, but it seems not improbable that it plays an important role in the synthesis of DNA. Since this process is highly radiosensitive, we undertook an investigation of the radiosensitivity of nuclear RNA. The latter was fractionated with 1 *M* NaCl, the heterogeneity of nuclear RNA now being well established by several authors[1–4]. Reports on the radiosensitivity of nuclear RNA have already been published[5–8] but in these studies no fractionation was attempted.

In this communication we shall briefly report the results of experiments concerning the metabolism and radiosensitivity of total and fractionated nuclear RNA from liver and thymus.

Groups of 5 young adult male albino rats, weighing 150 g, were irradiated in a perspex cage. Control groups consisted of 5 non-irradiated rats. All further procedures were carried out in exactly the same manner for the irradiated and the control group. The dose was 700 R in the experiments with liver tissue and 300 R with thymus tissue. In the experiments on liver nuclear RNA, the rats 4 h after irradiation were injected intravenously with 0.5 ml of neutralized 0.1 % Na_2HPO_4 containing 200 μC $Na_2H^{32}PO_4$, and killed 3.5 h later. In the studies on thymus nuclear RNA 200 μC

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; CMP, cytidine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate.

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TABLE I

THE EFFECT OF TOTAL-BODY IRRADIATION ON THE INCORPORATION OF LABELLED INORGANIC PHOSPHATE INTO NUCLEAR RNA

The irradiation dose was 700 R in the experiments with liver tissue and 300 R in those with thymus tissue.

	Liver RNA								*Thymus RNA*							
	Total nuclear RNA				*n-RNA I*				*Total RNA*				*n-RNA I*			
	specific activity (counts/min/μmole)		*r.s.a.* × 10³*		*specific activity (counts/min/μmole)*		*r.s.a.* × 10³*		*specific activity (counts/min/μmole)*		*r.s.a.* × 10³*		*specific activity (counts/min/μmole)*		*r.s.a.* × 10³*	
	irr.	*contr.*	*irr.*	*contr.*	*irr.*	*contr.*	*irr.*	*contr.*	*irr.*	*contr.*	*irr.*	*contr.*	*irr.*	*contr.*	*irr.*	*contr.*
CMP	2330	2050	285	225	1240	2780	150	305	415	955	350	775	425	1240	360	1000
AMP	4020	4000	490	435	1460	3180	180	350	910	2430	775	1935	700	1825	600	1485
GMP	3150	2540	385	290	1140	2860	140	315	605	1550	510	1260	420	1140	355	930
UMP	2000	2170	245	220	1440	3080	175	340	590	1340	500	1090	605	1300	510	1050
inorg. phosphate	8170	9110							1180	1230						

* The relative specific activity of the nucleotides, representing the quotient of the specific activities of the nucleotides and the nuclear inorganic phosphate.

$Na_2H^{32}PO_4$ was similarly injected 0.5 h after irradiation and the glands were excised 3 h later. Nuclei were isolated according to the method of HOGEBOOM *et al.*[9]. The nuclear suspensions were divided into two parts. One portion was centrifuged and the pellet was washed as described by OSAWA *et al.*[10] (total nuclear RNA). The other portion of the nuclear suspension was centrifuged and the pellet was homogenized with 20 vol. 1 *M* NaCl, pH 6.5. The suspension was shaken for 2 h and then centrifuged for 20 min at 15,000 rev./min. The precipitate was washed[10] (*n*-RNA II). An equal volume of 96 % ethanol was added to the supernatant. The precipitate was collected by centrifugation and washed[10] (*n*-RNA I). The RNA nucleotides were isolated according to the method of OSAWA *et al.*[10] and separated by high-voltage electrophoresis. Nuclear inorganic phosphate was obtained from the first washing fluids (with 2 % $HClO_4$) from the nuclear preparations. The nucleotides in the extracts were removed with charcoal and inorganic phosphate was isolated and analysed as described by VAN BEKKUM[11].

The results of the experiments are shown in Table I. It will be seen that the labelling of *n*-RNA I is a radiosensitive process both in liver and thymus tissue. The effect of X-irradiation on the uptake of ^{32}P in total nuclear RNA is different for thymus and liver. We have not yet fully elucidated the effect of X-irradiation on the uptake of ^{32}P in the nucleotides of *n*-RNA II. So far, a slight increase in the uptake of ^{32}P in this RNA fraction was found in liver tissue, whereas in thymus the uptake was considerably reduced. The fact that the relative specific activity of some of the nucleotides from nuclear thymus RNA is greater than unity can be explained by assuming a relatively more rapid exchange of ^{32}P of the nuclear inorganic phosphate as compared with the exchange of ^{32}P of the RNA nucleotides during the period studied. On the other hand, the possibility cannot be excluded that one of the precursors of nuclear RNA is cytoplasmic inorganic phosphate, which has in our experiments a higher specific activity than nuclear inorganic phosphate.

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[1] W. S. VINCENT, *Science*, 126 (1957) 306.
[2] R. LOGAN, *Biochim. Biophys. Acta*, 26 (1957) 227.
[3] Y. HOTTA AND S. OSAWA, *Biochim. Biophys. Acta*, 28 (1958) 642.
[4] R. LOGAN AND J. N. DAVIDSON, *Biochim. Biophys. Acta*, 24 (1957) 196.
[5] R. M. S. SMELLIE, S. F. HUMPHREY, E. R. M. KAY AND J. N. DAVIDSON, *Biochem. J.*, 60 (1955) 177.
[6] A. H. PAYNE, L. S. KELLY AND C. ENTENMAN, *Proc. Soc. Exptl. Biol. Med.*, 81 (1952) 798.
[7] E. HARBERS AND CH. HEIDELBERG, *J. Biol. Chem.*, 234 (1959) 1249.
[8] A. SIBATANI, *Exptl. Cell Research*, 17 (1959) 131.
[9] G. H. HOGEBOOM, W. C. SCHNEIDER AND M. J. STRIEBICH, *J. Biol. Chem.*, 196 (1952) 111.
[10] S. OSAWA, K. TAKATA AND Y. HOTTA, *Biochim. Biophys. Acta*, 28 (1958) 271.
[11] D. W. VAN BEKKUM, *Biochim. Biophys. Acta*, 25 (1957) 487.

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Response of tyrosine-α-ketoglutarate transaminase activity to ribonucleic acids

The induction of certain liver cytoplasmic enzymes, such as tyrosine-α-ketoglutarate transaminase[1] has been inferred to occur by virtue of increased net synthesis of the apoenzyme. This conclusion was drawn from a demonstration of induction of TAKT *in vivo* in the relative absence of coenzyme[2]. Additional indirect information emphasizes the importance of exogenous protein for the maintenance of TAKT activity[3]. To the present time, stimulation of TAKT activity following injection of L-tyrosine and/or hydrocortisone[1] has not been related directly to RNA, a relationship which would be expected to follow from investigations into the mechanism of protein synthesis in animal tissues. However, increased ^{32}P incorporation into RNA has been observed to follow induction of tryptophan peroxidase[4], and increased incorporation of labelled valine into partially purified fractions containing tryptophan peroxidase has been reported following induction of this enzyme[5].

In this laboratory it was considered that total liver RNA present at the peak of induction (5 h after injection of inducer) might contain a high-molecular-weight fraction which could, by itself, accomplish TAKT induction. It was found, instead, that total rat liver RNA prepared by known methods[6,7], which was soluble in water, accomplished the induction *in vivo*. Furthermore, induction was accomplished in a similar fashion following intraperitoneal injection of highly purified, highly polymerized RNA fractions from yeast, following injection of several commercial RNA preparations known to contain chiefly low-molecular-weight components, and finally, following injection of synthetic mixtures of 2′,3′-nucleotides simulating the ratios found in RNA. Peak stimulation of TAKT activity was observed 4 h after injection of RNA in each case (Table I). The optimal concentration of RNA 4 h after injection is signified by a sigmoid dependence curve which levels off at 20 mg/100 g body weight. These experiments were controlled by injections of equal volumes of saline, or by injection of an equal mass of heparin, or by the use of untreated animals. Slight stimulation was obtained from saline or heparin control reactions compared to the untreated controls. Heparin produced a higher response than saline, yet it was not considered to be comparable to RNA or nucleotide mixtures. The stimulation of TAKT activity by RNA appears to be mediated by the adrenal gland (or hydrocortisone), but this dependence is not absolute when nucleotide mixtures replace RNA in adrenalectomized animals. Stimulation is often found in the latter case, although it is not equivalent to the level found when the animal is intact or when replacement with hydrocortisone is instituted. Moreover, commercial sources of DNA have been poorly active in stimulating TAKT activity in whole animals. These findings emphasize the importance of studying the differential rates of breakdown of RNA and DNA in the peritoneal fluid. Studies with creatine phosphate and ATP ± glucose indicate that RNA does not cause the stimulation of TAKT activity by providing energy sources in the form of nucleotide derivatives.

In adrenalectomized animals the greatest stimulation of TAKT activity 4 h after injection is observable when hydrocortisone, RNA or the nucleotide mixture, and essential amino acids are supplied simultaneously through the intraperitoneal route.

Abbreviations: TAKT, tyrosine-α-ketoglutarate transaminase; ATP, adenosine triphosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

TABLE I

EFFECTS OF YEAST RNA PREPARATIONS ON RAT-LIVER TAKT ACTIVITY AS A FUNCTION OF TIME AFTER INTRAPERITONEAL INJECTION

30 mg/100 g body wt. purified high-molecular-weight yeast RNA.

Time after injection (h)	*Number of animals*	*Average body weight (g)*	*ANN*[*] $\times 10^8$	*Average TAKT activity*[**]			
				per g liver	*per 100 g body weight*	*per organ weight*	*per ANN* $\times 10^{-8}$
0.5							
Untreated	3	370	1.4	13.9	3.8	226	9.9
Heparin	3	344	1.9	16.4	4.8	283	8.6
RNA	3	366	2.1	14.8	4.0	247	7.0
2.0							
Untreated	3	312	1.4	13.7	4.4	169	10.1
Heparin	3	292	1.8	16.9	5.8	207	9.7
RNA	3	292	1.9	45.7	15.6	537	24.0
4.0							
Untreated	4	256	1.7	7.2	2.8	82	4.2
Heparin	4	288	2.7	11.4	3.9	124	4.2
RNA	4	278	2.2	101.0	36.2	1120	46.0
5.0							
Untreated	4	313	1.8	13.5	4.2	205	7.6
Heparin	8	280	2.1	22.7	7.8	313	10.5
RNA	12	289	2.5	86.5	31.1	1160	37.8
17.0							
Untreated	3	244	2.3	18.2	7.4	170	8.1
Heparin	3	256	2.3	10.7	4.2	129	4.8
RNA	3	260	2.0	16.2	6.2	181	8.1

[*] ANN = average number of liver nuclei/g determined by the method of BASS *et al*[8].

[**] Change in absorbancy at 310 mμ/10 min in a standard assay system[1] at 25° using 0.3 ml of a supernatant preparation. Total volume, 3.5 ml.

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[1] E. C. C. LIN AND W. E. KNOX, *Biochim. Biophys. Acta*, 26 (1957) 85.
[2] E. C. C. LIN, M. CIVEN AND W. E. KNOX, *J. Biol. Chem.*, 233 (1958) 1183.
[3] G. LITWACK, P. S. EBERT, S. YANKELOWITZ AND M. W. TAYLOR, *Enzymologia*, 20 (1959) 183.
[4] P. FEIGELSON, M. FEIGELSON AND C. FANCHER, *Biochim. Biophys. Acta*, 32 (1959) 133.
[5] P. GROS, J. COURSAGET AND G. P. TALWAR, *Bull. soc. chim. Belges*, 65 (1956) 107.
[6] K. S. KIRBY, *Biochem. J.*, 64 (1956) 405.
[7] A. GIERER AND G. SCHRAMM, *Nature*, 177 (1956) 702.
[8] A. D. BASS, A. H. MCARDLE AND J. W. GRISHAM, *Science*, 128 (1956) 372.

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Enzymic formation of citramalate from acetyl-coenzyme A and pyruvate in *Pseudomonas ovalis* Chester, catalysed by "pyruvate transacetase"

Evidence for the enzymic interconversion of citramalate, and acetate plus pyruvate, has been obtained in studies with liver mitochondria[1], *Clostridium tetanomorphum*[2–4], *Pseudomonas* sp.[5] and *Chromatium*[6], but, to our knowledge, the enzyme catalysing this interconversion has not been characterized nor has the mechanism of this reaction been established.

Cell-free extracts of *Ps. ovalis* Chester, which had been grown aerobically on itaconate as sole carbon source, catalysed the anaerobic incorporation of isotope from [2-^{14}C]acetate into citramalate, if fortified with $MgCl_2$, ATP, CoA and pyruvate (Table I). The labelled product of this reaction was shown to be citramalate by co-chromatography with authentic citramalate in three solvent systems[7] and by co-electrophoresis on paper in 0.5 *M* pyridine–0.5 *M* acetic acid adjusted to pH 4.0. Cell-free extracts of the organism grown on acetate as carbon source also effected this reaction, but at a rate which was less than 23% of that catalysed by similar extracts of itaconate-grown cells. Since the rate of acetate activation in this experiment may have imposed a rate-limiting step, these values are only qualitative; however, they indicate that the quantities of the enzyme which catalyses citramalate formation, present in the cell extracts, are influenced by the nature of the carbon source for growth. [^{14}C]citramalate was also formed from [3-^{14}C]pyruvate and acetyl-coenzyme A[8] in the presence of a dialysed cell-extract from itaconate-grown *Ps. ovalis* Chester (Table II): again, the formation of citramalate required the presence of these reactants, and the amount of the material formed was equivalent to the amount of pyruvate removed. Since the cell extracts fortified with ATP, CoA and $MgCl_2$ also catalysed the formation of acetyl-coenzyme A and pyruvate from citramalate, this

TABLE I

INCORPORATION OF ^{14}C FROM [2-^{14}C]ACETATE INTO CITRAMALATE

The complete system contained, in the main compartment of Warburg manometer vessels, 100 µmoles potassium phosphate, pH 7.5; 5 µmoles KF; 10 µmoles $MgCl_2$; 0.2 µmole CoA; and a dialysed sonic extract of itaconate-grown *Ps. ovalis* Chester (containing 3 mg protein). The centre wells of the cups contained 400 µmoles KOH. The manometers were equilibrated at 30° under N_2, after which 10 µmoles ATP, 5 µmoles sodium pyruvate and 2 µmoles sodium [2-^{14}C]acetate (containing 10 µC of ^{14}C and giving $6 \cdot 10^5$ counts/min) were added from the side-arms. The cups were detached after 40 min and samples of the contents mixed with 3 vol. ethanol. The labelled citramalate formed was located and assayed, as previously described, by radioautography[7].

Contents of cup	*Radioactivity of citramalate (counts/min × 10^{-1})*
Complete system	25.6
CoA omitted	3.16
ATP omitted	0.89
Pyruvate omitted	0.75
Complete system, but enzyme boiled	0.00

Abbreviations: ATP, adenosine triphosphate; CoA and CoASH, coenzyme A.

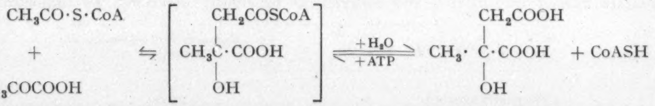
TABLE II

FORMATION OF [^{14}C]CITRAMALATE FROM ACETYL-COENZYME A AND [3-^{14}C]PYRUVATE

The complete system contained, in 2 ml, 100 μmoles potassium phosphate, pH 7.5; 10 μmoles $MgCl_2$; 0.55 μmole sodium [3-^{14}C]pyruvate (containing 2 μC ^{14}C); approx. 6 μmoles acetyl-coenzyme A, and dialysed sonic extract of itaconate-grown *Ps. ovalis* Chester (containing 2.7 mg protein). The mixture was incubated in a Warburg manometer under nitrogen for 40 min at 30°; the centre well of the cups contained 400 μmoles KOH to absorb any CO_2. After the incubation, the pyruvate content of samples of the cup contents was determined by the method of FRIEDEMANN AND HAUGEN[10]. Portions of the cup contents were also pipetted into 3 vol. ethanol and the quantities of labelled products assayed after two-dimensional radioautography, as previously described[7].

Contents of cup	*Pyruvate utilized*		*Citramalate formed*
	(by radioassay; mμmoles)	*(by keto-acid assay; mμmoles)*	*(by radioassay; mμmoles)*
Complete system	386	386	380
Acetyl-coenzyme A omitted	6	7	10
Complete, but enzyme boiled	0	0	0

reaction is reversible and indeed appears to favour citramalate cleavage rather than its formation. By analogy with other enzymes catalysing the transfer of acyl groups, we propose, in the nomenclature of DIXON AND WEBB[9], to describe the enzyme catalysing this reaction (i) as "pyruvate transacetase".



$$\begin{array}{c} CH_3CO\cdot S\cdot CoA \\ + \\ CH_3COCOOH \end{array} \leftrightharpoons \left[\begin{array}{c} CH_2COSCoA \\ | \\ CH_3C\cdot COOH \\ | \\ OH \end{array}\right] \underset{+ATP}{\overset{+H_2O}{\rightleftharpoons}} CH_3\cdot \begin{array}{c} CH_2COOH \\ | \\ C\cdot COOH \\ | \\ OH \end{array} + CoASH \qquad (i)$$

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[1] J. ADLER, S. F. WANG AND H. A. LARDY, *J. Biol. Chem.*, 229 (1957) 865.
[2] J. T. WACHSMAN, *J. Biol. Chem.*, 223 (1956) 19.
[3] H. A. BARKER, R. M. WILSON AND A. MUNCH-PETERSEN, *Federation Proc.*, 16 (1957) 151.
[4] A. MUNCH-PETERSEN AND H. A. BARKER, *J. Biol. Chem.*, 230 (1958) 649.
[5] V. BRIGHTMAN AND W. R. MARTIN, *Bacteriol. Proc.*, (1960) p. 162.
[6] M. LOSADA, A.V. TREBST, S. OGATA AND D. I. ARNON, *Nature*, 186 (1960) 753.
[7] H. L. KORNBERG, *Biochem. J.*, 68 (1958) 535.
[8] E. J. SIMON AND D. SHEMIN, *J. Am. Chem. Soc.*, 75 (1953) 2520.
[9] M. DIXON AND E. C. WEBB, *Enzymes*. Longmans, Green & Co., London, 1958, p. 217.
[10] T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.

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Location of butyric acid in bovine milk triglycerides

Investigations in several laboratories have shown that the volatile fatty acids of ruminant milk fat are synthesized in the mammary gland from acetate and β-hydroxybutyrate[1,2]. GLASCOCK, DUNCOMBE AND REINUS[3] have reported that the formation of the volatile fatty acids by the chain shortening of the unsaturated acids of the blood glycerides occurred only to a negligible extent. Substitution of a part of the unsaturated acids of the precursor triglycerides by the volatile acids has been suggested by HILDITCH[4] to account for the characteristic glyceride pattern of the milk fat.

Determination of the location of the volatile fatty acids in the ruminant-milk triglycerides was expected to provide a clue regarding the mechanism of their incorporation in the milk fat. Accordingly, advantage was taken of the specificity of pancreatic lipase for the fatty acids attached to the α-carbon atoms of glycerol[5]. Butyric acid was considered as typifying the short-chain fatty acids.

Butter fat, the molar butyric acid content of which was determined by the chromotographic procedure of KEENEY[6], was subjected to the action of pancreatic lipase. At the end of the incubation period the reaction was stopped by lowering the pH to 2.0 with 1 *M* HCl. The free fatty acids liberated and the glycerides remaining were extracted with alcohol–ether (1:2) mixture. The extract was washed free of the fatty acids with 1 % Na_2CO_3, the excess of Na_2CO_3 was washed out and the neutral glycerides were isolated. The molar proportion of butyric acid in this fraction was determined as before. The data are shown in Table I. It is seen that there is a significant loss of butyric acid from the glycerides. This finding shows that the acid

TABLE I

BUTYRIC ACID CONTENT OF BUTTER FAT GLYCERIDES AFTER LIPASE ACTION

2.0 g of fat (butyric acid, 10.2 molar %) were emulsified with 0.15 g sodium glycotaurocholate in tris(hydroxymethyl)aminomethane buffer, 2 ml of 45 % $CaCl_2$ was added and pH adjusted to 7.0. The mixture was incubated with 0.15 g pancreatic lipase at 39° for 3 min with vigorous stirring.

Expt.	*Volume of the medium (ml)*	*Molar % of butyric acid in the resulting glycerides*
1	80	4.3
2	160	3.2
3	360	2.4

TABLE II

FRACTIONATION OF THE GLYCERIDES AFTER LIPASE ACTION AND THEIR BUTYRIC ACID CONTENT

The procedure was the same as described under Table I.

Glycerides	*% recovered*	*Molar % of butyric acid*
Tri-	48.7	4.25
Di-	36.1	5.16
Mono-	11.9	0.0
Total	96.7	

is located predominantly at the α-carbon atoms of the glyceride. The greater loss of the acid with increasing dilution is undoubtedly a reflection of the inhibition of the exchange reaction between long-chain glyceride fatty acids and the free acids of the medium[5].

In further experiments the glyceride fraction isolated after lipase action was fractionated into mono-, di- and triglycerides by the procedure of QUINLIN AND WEISER[7] and the butyric acid content was determined in each species. The data obtained in one experiment are presented in Table II. Each of the experiments with varying incubation periods and enzyme and substrate concentrations yielded monoglycerides with no detectable butyric acid.

These results show that the butyric acid in the bovine milk fat is attached exclusively at the α-carbon atoms of the glyceride. This and the negligible exchange of the lower fatty acids between the different species of glycerides in the presence of lipase would suggest that they are incorporated into α, β-diglycerides, most probably by the mechanism suggested by WEISS, KENNEDY AND KIYASU[8].

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[1] G. POPJÁK, T. H. FRENCH AND S. J. FOLLEY, *Biochem. J.*, 48 (1951) 411.
[2] S. KUMAR, S. LAKSHMANAN AND J. C. SHAW, *J. Biol. Chem.*, 234 (1959) 754.
[3] R. F. GLASCOCK, W. G. DUNCOMBE AND L. R. REINUS, *Biochem. J.*, 62 (1956) 535.
[4] T. P. HILDITCH, *Symposia Biochem. Soc.*, 9 (1952) 63.
[5] B. BERGSTROM, *Biochim. Biophys. Acta*, 13 (1954) 491.
[6] M. KEENEY, *J. Assoc. Official Agr. Chem.*, 39 (1956) 212.
[7] P. QUINLIN AND H. J. WEISER, JR., *J. Am. Oil Chem. Soc.*, 35 (1958) 325.
[8] S. B. WEISS, E. P. KENNEDY AND J. Y. KIYASU, *J. Biol. Chem.*, 235 (1960) 40.

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L-Xylulokinase and L-xylulose 5-phosphate–L-ribulose 5-phosphate 3-epimerase in *Aerobacter aerogenes*

Growth of *Aerobacter aerogenes* on L-xylose evokes the formation of a cobalt-activated isomerase which produces L-Xu fom L-xylose[1], and two additional new enzymes, a kinase and a 3-epimerase, which effect the further metabolism of L-Xu. The L-xylulokinase has been purified 500-fold from cell extracts by protamine, ammonium sulfate, alumina Cγ, and DEAE-cellulose fractionations. Of the four ketopentoses, only L-Xu was phosphorylated. The product of L-Xu phosphorylation was prepared by incubating 1 mmole L-Xu, 1 mmole ATP, 100 μmoles NaGSH, 80 μmoles ethylenediaminetetraacetate, and 2 mmoles $MgCl_2$ with 633 units kinase* (before DEAE-

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Abbreviations: Xu, xylulose; Ru, ribulose; –P, phosphate; NaGSH, sodium glutathione; DEAE-cellulose, diethylaminoethylcellulose; ATP, adenosine triphosphate; TPN, triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

* The pH was maintained at 6.8 to 7.5 with 0.1 N NaOH. 1 unit = 1 μmole phosphorylated/h.

cellulose). After 2 h, the phosphorylation product was isolated as the barium salt (143 mg) by standard methods[2]. The kinase product, with a pentose to phosphate ratio of 0.94, acted as substrate for L-Ru-5-P 4-epimerase[3], but not for a mixture of phosphoriboisomerase, D-Ru-5-P 3-epimerase, and D-Xu-5-P phosphoketolase*. After incubation with purified acid phosphatase and chromatography on Dowex-1-borate, two peaks were eluted. Peak 1 (70 % of the ketose) was identified as Xu and Peak 2 behaved identically with authentic Ru (Table I).

TABLE I

IDENTIFICATION OF FREE KETOSE FROM L-XYLULOKINASE PRODUCT

Tests	*Peak 1*	*Xylulose*	*Peak 2*	*Ribulose*
Yield, μmoles	101	—	42	—
Dowex-1-borate, elution concn. (*M*)	0.02	0.02	0.03	0.03
Orcinol, 540/670 mμ	0.40	0.42–0.48	0.87	0.79–0.99
Cysteine-carbazole, min for maximum color	>60	>60	<15	<145
Chromatography*				
R_F	0.57	0.57	0.54	0.54
Dimethylphenaline**	Gray	Gray	Gray-Brown	Gray-Brown
Orcinol-trichloroacetic acid	Rose***	Rose***	Pink §	Pink §

* butanol-pyridine-water (6:4:3)[4].
** N,N-Dimethyl-*p*-phenylenediamine.
*** Rose fluorescence.
§ Orange fluorescence.

Peak 1 acted as substrate for TPN-specific xylitol (L-Xu) dehydrogenase[4], but not for a specific D-arabitol (D-Xu) dehydrogenase[5], or for a purified ribitol (D-Ru) dehydrogenase[5,6]. Peak 2 reacted with L-arabinose (L-Ru) isomerase[7], but not with D-arabinose (D-Ru) isomerase, or the pentitol dehydrogenases. Therefore, an enzymic conversion of L-Xu-P to L-Ru-5-P was indicated.

The reverse reaction, L-Ru-5-P to L-Xu-P, was observed by incubating L-Ru-5-P (prepared with purified L-Ru kinase[2]) for 6 h with 43 units of the same L-Xu kinase fraction at pH 8.5. A disappearance of L-Ru-5-P was observed**, and D-ribose 5-phosphate, D-Ru-5-P and D-Xu-5-P were not produced. After phosphatase treatment, chromatography on Dowex-1-borate yielded two ketopentose peaks in elution positions for Xu (68 %) and Ru (32 %). As above, Peak 1 reacted in the chemical and enzymic tests as L-Xu, whereas Peak 2 was identified as L-Ru. D-Ru was not detected.

The conversion of L-Xu-P to L-Ru-5-P was also observed in a spectrophotometric assay for L-Ru-5-P[3,8]. With excess L-Ru-5-P 4-epimerase, phosphoketolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase, the formation of L-Ru-5-P may be observed as DPNH oxidation. As shown in Fig. 1, DPNH was oxidized rapidly by the complete system, but was oxidized only slowly when either ATP, L-Xu, or 4-epimerase were omitted.

Because L-Ru-5-P is both formed from and converted to L-Xu-P, it is likely that L-Xu is phosphorylated at carbon 5. The conversion of L-Xu-5-P to L-Ru-5-P, thus, is analogous to the D-Ru-5-P 3-epimerase reaction. However, the L-Xu kinase fraction does not contain D-Ru-5-P 3-epimerase, and purified D-Ru-5-P 3-epimerase from yeast does not interconvert the L-isomers. Therefore, it is concluded that a new L-Xu-5-P (or L-Ru-5-P) 3-epimerase catalyzes the interconversion of L-Xu-5-P and L-Ru-5-P. Since L-xylose-grown cells also contain L-Ru-5-P 4-epimerase, the metabolic sequence is considered to be: L-Xylose → L-Xu → L-Xu-5-P → L-Ru-5-P → D-Xu-5-P. This route is distinct from the mammalian system wherein two xylitol dehydrogenases mediate the conversion of L-Xu to D-Xu, which is then phosphorylated[9,10].

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* Under conditions in Fig. 1 except that L-Ru-5-P 4-epimerase was omitted.
** DPNH oxidation under conditions in Fig. 1.

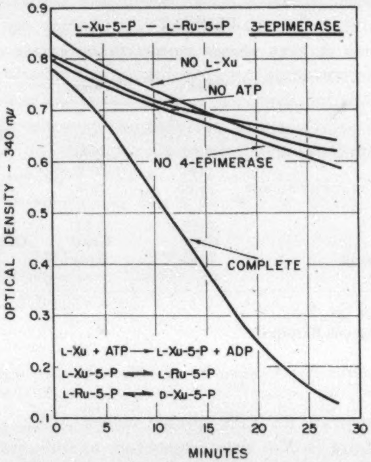

Fig. 1. Formation of L-Ru-5-P from L-Xu-5-P by L-Xu-5-P (L-Ru-5-P) 3-epimerase. The reaction mixture contained in 0.3 ml crystalline α-glycerophosphate dehydrogenase (containing triose phosphate isomerase), purified L-Ru-5-P 4-epimerase[7], purified D-Xu-5-P phosphoketolase[8] (each in excess), 8 units of kinase fraction containing L-Ru-5-P 3-epimerase, and the following in μmoles: glycylglycine, pH 7.5, 15; $MgCl_2$, 1; DPNH, 0.05; sodium arsenate, 2; NaGSH, 3; thiamine pyrophosphate chloride, 0.1; ATP, 3; and L-Xu, 2.9.

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[1] R. L. Anderson, *Federation Proc.*, 19 (1960) 82.
[2] F. J. Simpson and W. A. Wood, *J. Biol. Chem.*, 230 (1958) 473.
[3] M. J. Wolin, F. J. Simpson and W. A. Wood, *J. Biol. Chem.*, 232 (1958) 559.
[4] J. Hickman and G. Ashwell, *J. Biol. Chem.*, 234 (1959) 758.
[5] W. A. Wood and J. V. Tai, *Bacteriol. Proc.*, p. 99 (1958).
[6] H. J. Fromm, *J. Biol. Chem.*, 233 (1958) 1049.
[7] F. J. Simpson, M. J. Wolin and W. A. Wood, *J. Biol. Chem.*, 230 (1958) 457.
[8] D. P. Burma and B. L. Horecker, *J. Biol. Chem.*, 231 (1958) 1053.
[9] S. Hollmann and O. Touster, *J. Biol. Chem.*, 225 (1957) 87.
[10] J. Hickman and G. Ashwell, *J. Am. Chem. Soc.*, 78 (1956) 6209.

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The isolation of D-valine from *Penicillium chrysogenum*

During the course of work on the identification of possible intermediates in penicillin biosynthesis, we have isolated, from the mycelium of *Penicillium chrysogenum*, several compounds containing cystine/cysteine and valine. Since these amino acids are precursors of penicillin[1], intermediates in the biosynthetic pathway must contain then in some form. The valine moiety in the final penicillin molecule has the D-configuration although L-valine is the probable ultimate precursor[2,3]. It was of interest, therefore, to examine the configuration of the valine derived from the above compounds.

Mycelium of *P. chrysogenum* strain Wis. 51–20F3 was grown in the medium of JARVIS AND JOHNSON[4], and harvested and washed as previously described[5]. The washed mycelium was resuspended in 0.01 *M* phosphate buffer, pH 7.0, containing 0.025 g/l potassium phenylacetate (100 g wet mycelial pad in 800 ml of liquid in ten 500-ml flasks), and shaken for 1 h at 24°. These conditions permit the continued synthesis of benzylpenicillin[5]. The mycelium was then washed again with three changes of water at 2°. The aim of this washing at reduced temperature was to minimize further metabolism of any accumulated intracellular intermediates.

After the final washing, the mycelium was subjected to mild acid hydrolysis (800 ml 1 *N* HCl, 56°, 2 h), filtered, and the cooled filtrate extracted with diethyl ether. Some purification of the extract was effected by extraction into aqueous buffer at pH 7 and back into ether at pH 2. The extract was then evaporated to about 0.5 ml, and portions were fractionated by paper chromatography (Whatman No. 1 paper, butanol–acetic acid–water (100:24:100, v/v/v), samples neutralized with ammonia vapor before running), and the paper sprayed with ammoniacal $AgNO_3$. The most prominent spots which appeared were colorless against the brown background and had R_F values of 0.61, 0.79 and 0.86; they gave no color reaction with ninhydrin.

Complete hydrolysis of the substances eluted from these spots yielded the same mixture of amino acids in each case, as judged by conventional paper chromatography and spraying with ninhydrin. The amino acids appeared to be valine, cystine/cysteine, glycine and leucine. Although attempts were made to demonstrate phenylacetic acid—the side-chain precursor of penicillin—in the hydrolysates, this has not yet been accomplished to our satisfaction. It is presumed that the three substances yielding amino acids on further hydrolysis are peptides.

The yield of valine from 100 g of mycelial pad (10 g dry wt.) was approximately 50 µg. Because of the small quantities of material available, the following micro methods were used to examine the configuration of the isolated valine:

1. *Paper chromatography*. Conventional chromatography, with the conditions detailed above, showed that D- and L-valine differed slightly but consistently in their R_F values.

2. *Microbiological assay*. Species of lactobacilli differ in their growth response to the optical isomers of amino acids[6]. The responses (acid production after 3-days' incubation) of *Lactobacillus arabinosus* 17–5 and *L. fermenti* ATCC 9338 to the isolated valine and to graded amounts of D- and L-valine were measured in a synthetic valine assay medium.

3. D-*Amino acid oxidase*. The isolated valine was treated with crude D-amino

acid oxidase (Sigma Chemical Co.) of known activity, in a 0.02 *M* pyrophosphate buffer, pH 8.3. The ammonia liberated during 5-min incubation was determined colorimetrically with Nessler's reagent. Known quantities of D- and L-valine were similarly treated.

4. *Ninhydrin reaction*. The total valine was determined by the quantitative ninhydrin reaction[7].

The results of these four determinations are shown in Table I.

TABLE I

RESPONSES OF L-, D- AND ISOLATED VALINE TO VARIOUS TESTS

Method:	*1*	*2*		*3*	*4*
	R_F *(Butanol-acetic acid)*	*L. arabinosus*	*L. fermenti*	*D-amino acid oxidase*	*Ninhydrin*
L-valine	0.49	+	+	—	+
D-valine	0.51	—	+	+	+
Isolated valine	0.51	—	+	+	+

Quantitatively, the positive responses of the isolated valine were similar; for example, a single sample assayed 13 (± 3) μg, 12 (± 3) μg and 12 (± 1) μg by methods 2, 3 and 4 respectively, indicating the absence of L-valine within experimental error. Thus all the valine in each of the three peptides was D-valine.

The presence of peptides of D-valine in a penicillin-producing organism might be expected, in view of the structure and origin of the penicillin molecule. However, this is the first report of the isolation of D-valine from the mycelium of *P. chrysogenum*, where it has been claimed to be absent[8]. The existence of intracellular, ether-soluble peptides containing cystine/cysteine and D-valine has important implications for theories of the mechanism of penicillin biosynthesis. These will be discussed in a more detailed account to be published elsewhere.

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[1] H. R. V. Arnstein and P. T. Grant, *Biochem. J.*, 57 (1954) 353, 360.
[2] C. M. Stevens, E. Inamine and C. W. Delong, *J. Biol. Chem.*, 219 (1956) 405.
[3] H. R. V. Arnstein and H. Margreiter, *Biochem. J.*, 68 (1958) 339.
[4] F. G. Jarvis and M. J. Johnson, *J. Bacteriol.*, 59 (1950) 51.
[5] F. H. Grau and W. J. Halliday, *Biochem. J.*, 69 (1958) 205.
[6] S. A. Koser and J. L. Thomas, *J. Bacteriol.*, 73 (1957) 477.
[7] S. Moore and W. H. Stein, *J. Biol. Chem.*, 176 (1948) 367.
[8] C. M. Stevens, P. E. Halpern and R. P. Gigger, *J. Biol. Chem.*, 190 (1951) 705.

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Présence d'une vasotocine dans la neurohypophyse de la grenouille *(Rana esculenta L.)*

Les hormones neurohypophysaires du poulet responsables des activités ocytocique et pressique ont été récemment purifiées[1, 2] et trois substances ont pu être mises en évidence alors que chez les mammifères deux hormones seulement, *l'ocytocine* et la *vasopressine*, rendent compte des activités mentionnées. Il a paru intéressant d'étudier les principes actifs des vertébrés inférieurs chez lesquels la régulation du métabolisme hydrominéral pose des problèmes différents de ceux des mammifères et des oiseaux. L'existence d'un facteur nouveau chez les vertébrés à sang froid a été suggérée par PICKERING ET HELLER[3], SAWYER *et al.*[4], et MAETZ *et al.*[5,6] sur la base de données pharmacologiques. L'hypophyse de grenouille se prêtant aisément à la séparation des lobes "antérieur" et "postérieur", ce matériel a été choisi en premier lieu.

La dissection de 20,000 glandes a fourni 2 g de poudre acétonique post-hypophysaire titrant environ 1 unité U.S.P. ocytocique ou vasopressique/mg. L'activité "natriférique", c'est-à-dire l'action sur le transport actif du sodium à travers la peau isolée de grenouille, a été mesurée par la méthode de USSING[7] modifiée par MOREL *et al.*[8], en prenant comme référence le standard post-hypophysaire U.S.P., qui par convention possède 2 unités natriférique/mg. La poudre post-hypophysaire de grenouille titre environ 10 unités natriférique/mg.

Deux préparations ont été effectuées en utilisant chaque fois 1 g de matière première. La purification a été entreprise en adsorbant spécifiquement les hormones sur une protéine, la *neurophysine* du mouton[9]. L'extraction de la poudre par H_2SO_4 dilué, l'addition de neurophysine et la préparation du complexe par NaCl sont conduites de la façon antérieurement décrite pour la purification des hormones du poulet[1]. A ce stade environ 70–80 % des trois activités sont recueillies. Le complexe est dissocié par l'acide trichloracétique à 5 % qui précipite la neurophysine mais laisse les principes actifs en solution (50 à 70 % des 3 activités); après élimination de l'acide, les hormones sont chromatographiées sur une colonne d'Amberlite IRC-50 en utilisant

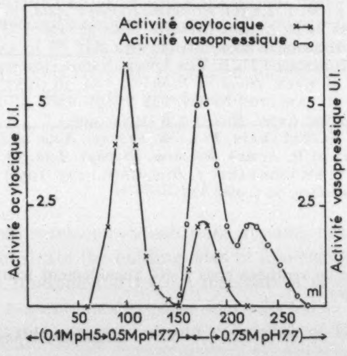

Fig. 1. Chromatographie des hormones neurohypophysaires de la grenouille sur Amberlite IRC-50: les hormones sont adsorbées au sommet d'une colonne de résine forme H (0.9 × 10 cm); la colonne est ensuite équilibrée avec un tampon acétate d'ammonium 0.1 *M* à pH 5.0 et le gradient est successivement réalisé avec des solutions 0.5 *M* et 0.75 *M* à pH 7.7.

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un gradient de pH et de force ionique au moyen de tampons acétate d'ammonium. La Fig. 1 indique les résultats en ce qui concerne les activités ocytocique et pressique.

On constate la présence d'au moins trois substances actives. La première sortie de la colonne possède la majeure partie de l'activité ocytocique récupérée. La seconde possède les deux activités ocytocique et pressique. La troisième ne possède pratiquement que l'activité pressique et sa position sur le chromatogramme correspond à celle de l'arginine-vasopressine des mammifères. Les tubes contenant la deuxième substance sont réunis et une fraction du matériel est rechromatographiée sur une colonne de carboxyméthylcellulose (1.5 × 22 cm) en utilisant un gradient de pH et de force ionique au moyen de tampons acétate d'ammonium (0.02 M, pH 5.2 à 0.2 M, pH 7.0). On recueille des fractions de 1 ml et on dose les trois activités pressique, ocytocique et natriférique dans les tubes. On constate une coïncidence des courbes des 3 activités qui plaide en faveur de l'homogénéité du matériel. L'hydrolyse du produit par HCl 6 N fournit les acides aminés suivants: $(\mathrm{Cys})_2$, Tyr, Ileu, Asp, Glu, Pro, Arg, Gly avec une faible quantité d'Ala. La substance isolée correspond par sa composition en acides aminés, sa position sur le chromatogramme d'Amberlite IRC-50 et ses propriétés biologiques à une nouvelle hormone déjà obtenue à partir de la posthypophyse de poulet[2]; sa composition paraît identique à celle d'un analogue de la vasopressine, *l'arginine-vasotocine*, synthétisé par KATSOYANNIS ET DU VIGNEAUD[10]. D'autre part, étudiées sur différents tests, les propriétés biologiques du produit isolé se sont avérées comparables à celles de l'arginine-vasotocine de synthèse*. Ces résultats sont en accord avec les observations pharmacologiques de SAWYER[11] suggérant l'existence de cette hormone chez les vertébrés inférieurs.

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[1] R. ACHER, J. CHAUVET ET M. T. LENCI, *Biochim. Biophys. Acta*, 38 (1960) 344.
[2] J. CHAUVET, M. T. LENCI ET R. ACHER, *Biochim. Acta*, 38 (1960) 571.
[3] B. T. PICKERING ET H. HELLER, *Nature*, 184 (1959) 1463.
[4] W. H. SAWYER, R. A. MUNSICK ET H. B. VAN DYKE, *Nature*, 184 (1959) 1464.
[5] J. MAETZ, F. MOREL ET B. RACE, *Biochim. Biophys. Acta*, 36 (1959) 317.
[6] J. MAETZ, F. MOREL ET B. LAHLOUH, *Nature*, 184 (959) 236.
[7] H. H. USSING, *Symposia Soc. Exptl. Biol. No.* 8 (1954), 407.
[8] F. MOREL, J. MAETZ ET CL. LUCARAIN, *Biochim. Biophys. Acta*, 28 (1958) 619.
[9] J. CHAUVET, M. T. LENCI ET R. ACHER, *Biochim. Biophys. Acta*, 38 (1960) 266.
[10] P. G. KATSOYANNIS ET V. DU VIGNEAUD, *J. Biol. Chem.*, 233 (1958) 1352.
[11] W. H. SAWYER, *Endocrinology*, 66 (1960) 112.

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COMPLEX FORMATION OF APO-ENZYME, COENZYME AND SUBSTRATE OF D-AMINO ACID OXIDASE

I. KINETIC ANALYSIS USING INDICATORS

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SUMMARY

1. It was found that both riboflavin-5′-monosulfate (FMS) and adenosine-5′-monosulfate (AMS) compete with FAD for binding with apo-D-amino acid oxidase but not with each other, which suggests the binding of the FMN as well as the AMP moieties of the FAD molecule with the apo-protein.

2. Using FMS and AMS as indicators, *p*-aminobenzoic acid (PABA) was found to be a competitor with the FMN part of FAD. It was considered likely that the phenylamino group affects the binding point of the FMN part of FAD with the apo-protein. The dissociation constant of the apo-protein–PABA complex calculated from the competition of PABA with FAD for the protein appeared to be $7.3 \cdot 10^{-3}$ *M*. In the presence of AMS, this dissociation constant decreased to $2.5 \cdot 10^{-3}$ *M*, *i.e.*, the affinity of PABA to the protein increased, which indicates an interaction between the two binding sites.

3. *p*-Chloromercuribenzoate (PCMB) was found to be a competitor of the AMP moiety of FAD. This action was attributed to the binding of the SH-reactive group of PCMB with the sulfhydryl group of the apo-protein, which suggests that the AMP moiety of FAD combined with sulfhydryl group of the apo-protein. The dissociation constant of the complex of PCMB and apo-protein was calculated to be $1.6 \cdot 10^{-7}$ *M*. In the presence of FMS, this constant decreased to $8.5 \cdot 10^{-8}$ *M*, also suggesting an interaction of the binding sites of the apo-protein.

INTRODUCTION

In order to elucidate the processes involved in the dynamic enzyme complex formation it is necessary to demonstrate the binding sites of apo-enzyme, coenzyme, and substrate, since these are fundamental to the mechanism of enzyme action. For the study of this subject, D-amino acid oxidase was selected as a tool. The reasons for the selection are that the apo-enzyme and coenzyme of this enzyme are instan-

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Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin-5′-monophosphate; AMP, adenosine-5′-monophosphate; FMS, riboflavin-5′-monosulfate; AMS, adenosine-5′-monosulfate; PABA, *p*-aminobenzoic acid; PCMB, *p*-chloromercuribenzoate.

taneously dissociable and associable with a rather large dissociation constant, and that the coenzyme FAD has a characteristic physico-chemical nature.

Concerning the binding of D-amino acid oxidase, previous work[1-5], especially the series on the mechanism of inhibition of this enzyme[3-5], provided some evidence. It has been noted by a model experiment and kinetic analysis of enzyme action[4] that the tyrosyl group of the apo-protein is responsible for the complex formation with the 3-NH group of the isoalloxazine ring of FAD, which is analogous to the old yellow enzyme reported by THEORELL AND NYGAARD[6].

At the same time, the phenylamino group of the benzene derivative was found to affect the binding site of the apo-protein with FAD[4], but the portion affected has not been studied.

On the other hand, the importance for the enzymic activity of the sulfhydryl group in D-amino acid oxidase apo-protein has been reported by several workers[7-10] but their conclusions about the binding site of the protein-sulfhydryl group were all different. SINGER AND BARRON[7], and SINGER[8] suggested that the protein-sulfhydryl group might be involved directly in the binding of the substrate. FRISELL AND HELLERMAN[9] concluded, however, that the enzyme-sulfhydryl group is not needed for the direct binding of the substrate, D-amino acids. KUBO *et al.*[10] were of the view that the protein-sulfhydryl group binds with the 3-NH group of FAD. Thus, opinions are divided about the binding of the sulfhydryl group of the apo-protein.

The indicator method previously reported for the study of the mechanism of inhibition[5] proved to be useful for solving these problems.

Results obtained by kinetic analyses for the purpose of demonstrating of the bindings involved in D-amino acid oxidase are reported in this paper. A preliminary note has already appeared[11].

MATERIALS

D-Amino acid oxidase protein, free from FAD, was prepared by the method of NEGELEIN AND BRÖMEL[12].

FAD was prepared by the method of YAGI *et al.*[13], as described previously.

FMS and AMS, kinetic indicators, were synthesized by the method of TAKAHASHI, YAGI, AND EGAMI[14], and that of EGAMI AND TAKAHASHI[15], respectively.

DL-Alanine, PABA, and PCMB were chemically synthesized samples.

METHOD

The oxidase activity was measured as described previously[3]. The dissociation constant of FAD with the apo-protein (K_f) and that of D-alanine with the apo-protein (K_s) were calculated to be $1.1 \cdot 10^{-7}\,M$ and $3.3 \cdot 10^{-3}\,M$, respectively, using the equation of Michaelis-Menten.

Assuming that an inhibitor inhibits the oxidase activity in competition with the coenzyme, FAD, for the apo-protein, the reaction velocity (v) can be shown as

$$v = \frac{Vf}{K_f(1 + i/K_{ip}) + f} \tag{1}$$

where K_{ip} is the dissociation constant of inhibitor–protein complex, f and i are the concentration of FAD and inhibitor, respectively, and V is the maximum velocity obtained in the presence of a large excess of FAD.

Rearranging eqn. (1) we obtain:

$$\frac{1}{v} = \frac{K_f\,(1 + i/K_{ip})}{Vf} + \frac{1}{V} \qquad (2)$$

K may be calculated from the slope of the straight line obtained by plotting $1/v$ against $1/f$ in the presence of a restricted amount of inhibitor (Method I).

K_{ip} may also be evaluated by the following equation, which is derived from the eqn. (1) and the Michaelis-Menten equation,

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\}\frac{i}{K_{ip}} \qquad (3)$$

where v_0 and v are the reaction velocity in the absence and presence of the inhibitor, respectively, with a restricted amount of FAD. The slope of the straight line obtained by plotting v_0/v against i refers to $(1 - v_0/V)/K_{ip}$.

If the reaction mixture contains two inhibitors, both of which compete with the same part of FAD (Case I), the reaction velocity can be shown as follows (see the formulation in the previous paper[5]):

$$v = \frac{Vf}{K_f\,(1 + i_1/K_1 + i_2/K_2) + f} \qquad (4)$$

where i_1 and i_2 are the concentration of two inhibitors, I_1 and I_2, and K_1 and K_2 are the dissociation constants of complexes (I_1-enzyme protein) and (I_2-enzyme protein), respectively.

On the other hand, if the reaction mixture contains two inhibitors one of which (I_1) competes with the FMN part of FAD and the other (I_2) competes with the AMP part of FAD, respectively, or in other words, inhibitors compete with FAD but not with each other, the reaction velocity can be shown as

$$v = \frac{Vf}{K_f\,(1 + i_1/K_1 + i_2/K_2 + i_1 i_2/K_1 K_3) + f}$$

or

$$v = \frac{Vf}{K_f\,(1 + i_1/K_1 + i_2/K_2 + i_1 i_2/K_2 K_4) + f} \qquad (5)$$

where K_3 is the dissociation constant of (I_1, I_2-enzyme protein) $\rightleftharpoons I_2 +$ (I_1-enzyme protein), and K_4 is that of (I_1, I_2-enzyme protein) $\rightleftharpoons I_1 +$ (I_2-enzyme protein). If no interaction takes place between the affinities of I_1-enzyme protein and that of I_2-enzyme protein, namely $K_3 = K_2$ and $K_4 = K_1$, eqn. (5) can be represented as

$$v = \frac{Vf}{K_f\,(1 + i_1/K_1 + i_2/K_2 + i_1 i_2/K_1 K_2) + f} \qquad (6)$$

From the eqns. (4), (5), or (6) and the Michaelis-Menten equation, the following equations can be derived

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2}\right) \qquad (7)$$

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_3}\right)$$

or

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_2 K_4}\right) \tag{8}$$

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_2}\right) \tag{9}$$

These equations show that the plots of v_0/v against the concentration of inhibitors give a straight line in Case I (eqn. (7)) and a second-order curve in Case II (eqn. (8) or (9)). Accordingly, if plots of v_0/v against i are on a straight line in the presence of both the inhibitor and FMS, and those are on a second-order curve in the presence of both the inhibitor and AMS, it may be concluded that the inhibitor competes with the FMN part of FAD. On the contrary, if plots of v_0/v are on a second-order curve in the presence of both the inhibitor and FMS, and on a straight line in the presence of both the inhibitor and AMS, it may be concluded that the inhibitor competes with the AMP part of FAD.

In this way, the behaviour of an inhibitor in its competition with FAD can be analyzed and the binding site of the apo-protein can also be studied.

RESULTS

Inhibitory action of FMS and AMS on D*-amino acid oxidase and their interaction on the oxidase protein*

The values of v_0/v plotted against the concentration of FMS or AMS were on a straight line as shown by Curves I and II in Fig. 1.

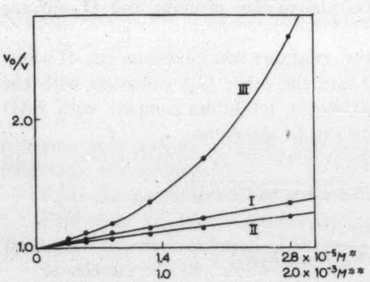

Fig. 1. Inhibitory action of FMS and AMS on D-amino acid oxidase in competition with FAD. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), a restricted amount of FAD ($1.7 \cdot 10^{-7}$ M, final concentration) and graduated concentrations of inhibitors.

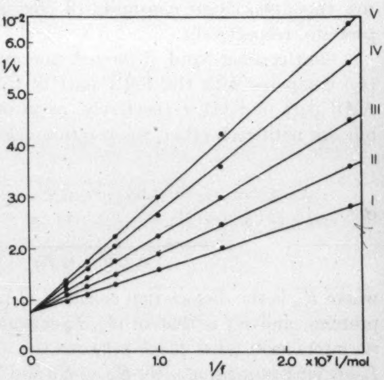

Fig. 2. Inhibitory action of FMS, AMS, PABA and PCMB on D-amino acid oxidase in competition with FAD. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), graduated concentrations of FAD as indicated in the figure, and a concentration of inhibitor as shown below. I: in the absence of inhibitor; II: in the presence of $1.25 \cdot 10^{-5}$ M (final concentration) of FMS; III: in the presence of $2.5 \cdot 10^{-3}$ M (final concentration) of AMS; IV: in the presence of $1.0 \cdot 10^{-2}$ M (final concentration) of PABA; V: in the presence of $2.8 \cdot 10^{-7}$ M (final concentration) of PCMB.

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* Indicates the concentration of FMS, and ** that of AMS. I: in the presence of FMS; II: in the presence of AMS; III: in the presence of both FMS and AMS. V was 132 μl O_2 uptake/30 min and v_0 was 80 μl O_2 uptake/30 min.

This result indicates that the inhibition is of single mechanism involving FAD. Method I was applied and the plots of $1/v$ against $1/f$ gave a straight line with the same intercept as that obtained by the plots of the experiment without any inhibitor, as shown in Fig. 2. From these results, it may be concluded that both FMS and AMS compete with FAD. The dissociation constant of the complex of FMS and the apo-protein, and that of AMS and the apo-protein were calculated to be $3.2 \cdot 10^{-5}$ M and $3.1 \cdot 10^{-3}$ M, respectively, from the slope of Curve I and Curve II in Fig. 1, and Curve II and Curve III in Fig. 2.

The presence of both FMS and AMS causes the plots of v_0/v to be second-order curve, as shown by Curve III in Fig. 1. Curve III shows that FMS and AMS do not compete with each other, but with the FMN and AMP part of FAD, respectively. By substituting the values of v_0/v into eqn. (8), K_3 and K_4 were calculated to be $6.6 \cdot 10^{-4}$ M and $6.7 \cdot 10^{-6}$ M, respectively. These results indicate that a positive interaction occurred between the binding portion of the apo-protein with the FMN part of FAD and that of the apo-protein with the AMP part of FAD, so that the affinity of each binding ($1/K_4$, $1/K_3$) becomes five times larger than the original affinity ($1/K_1$, $1/K_2$).

Analysis of inhibition by PABA of the oxidase, using kinetic indicators

As reported previously[4], it was demonstrated by methods I and II that PABA inhibits the oxidase in competition with FAD. The results are shown in Fig. 2, Curve IV, and in Fig. 3, Curve I. From these curves K_{ip} was calculated to be $7.3 \cdot 10^{-3}$ M.

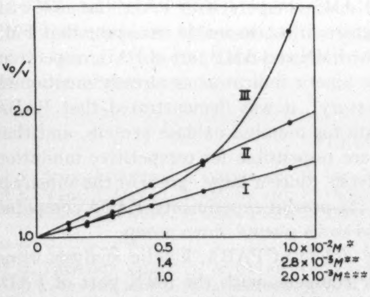

Fig. 3. Analysis of the inhibitory action of PABA on D-amino acid oxidase in competition with FAD, using kinetic indicators. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), a restricted amount of FAD ($1.7 \cdot 10^{-7}$ M, final concentration) and graduated concentration of inhibitors.

* Indicates the concentration of PABA, ** that of FMS, and *** that of AMS. I: in the presence of PABA; II: in the presence of both PABA and FMS; III: in the presence of both PABA and AMS. V was 132 μl O_2 uptake/30 min and v_0 was 80 μl O_2 uptake/30 min.

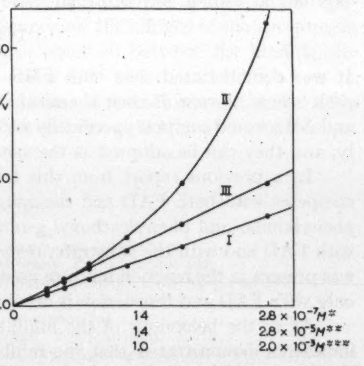

Fig. 4. Analysis of the inhibitory action of PCMB on D-amino acid oxidase in competition with FAD, using kinetic indicators. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), a restricted amount of FAD ($1.7 \cdot 10^{-7}$ M, final concentration) and graduated concentration of inhibitors.

* Indicates the concentration of PCMB, ** that of FMS, and *** that of AMS. I: in the presence of PCMB; II: in the presence of both PCMB and FMS; III: in the presence of both PCMB and AMS. V was 132 μl O_2 uptake/30 min and v_0 was 80 μl O_2 uptake/30 min.

In the presence of FMS and PABA, plots of v_0/v are on a straight line, as shown in Fig. 3, Curve II, which indicates that FMS and PABA compete with each other. On the other hand, plots of v_0/v in the presence of AMS and PABA are on a second-order curve, as shown in Fig. 3, Curve III, which indicates that AMS and PABA do not compete with each other. From these results, it is concluded that PABA competes with the FMN part of FAD.

Analysis of inhibition by PCMB of the oxidase, using kinetic indicators

It was observed through spectrophotometry and fluorimetry that PCMB does not form a complex with FAD. Method I in the presence of $2.8 \cdot 10^{-7}\,M$ of PCMB, a rate-limiting concentration of FAD, and excess DL-alanine ($0.15\,M$ in its final concn.) gave a straight line with the intercept $1/V$ as shown in Fig. 2, Curve V. From the slope of this line, K_{ip} was calculated to be $1.6 \cdot 10^{-7}\,M$. Plots of v_0/v against concn. of PCMB (Method II) were on a straight line with the intercept 1, as shown in Fig. 4, Curve I. The value of K_{ip} obtained from the slope of this line agreed with that mentioned above.

Plots of v_0/v against concns. of PCMB and FMS gave a second-order curve as shown in Fig. 4, Curve II, whereas in the presence of PCMB and AMS they gave a straight line as shown in Fig. 4, Curve III.

From these results, it may be concluded that PCMB combines with the apo-protein in competition with the AMP part of FAD.

DISCUSSION

It was demonstrated that both FMS and AMS compete with FAD, but not with each other. In view of their chemical structure, it is reasonable to expect that FMS and AMS would compete specifically with the FMN and AMP part of FAD, respectively, and they can be adopted as the specific kinetic indicators as already mentioned.

In a previous report from this laboratory[4], it was demonstrated that PABA competes with both FAD and the substrate for D-amino oxidase protein, and that phenylamino and phenylcarboxyl groups are responsible for competitive inhibition with FAD and with the substrate, respectively. Since a large excess of the substrate was present in the reaction mixture used in the present experiments, PABA competed only with FAD and this action is attributed to its phenylamino group.

As to the behaviour of the inhibitory action of PABA, kinetic analysis using indicators demonstrated that the inhibitor competes with the FMN part of FAD. In view of the chemical structure of FMN, it was assumed that the competition occurred between the phenylamino group of PABA and the 3-NH group of the isoalloxazine ring.

The inhibitory action of PCMB was also examined and it was found that PCMB competes with FAD to combine with the apo-protein, and that it competes with the AMP part of FAD.

Concerning the mechanism of inhibitory action of PCMB, SINGER AND BARRON[7] reported competitive inhibition of PCMB with the substrate and they suggested the combination of the protein-sulfhydryl group with the substrate.

However, the previous paper from this laboratory[4] demonstrated that the inhibitory action of benzoate or its derivatives in competition with the substrate can be attributed to their phenylcarboxyl group and that this type of inhibition is independent of other radicals in the benzene ring. Therefore, the inhibitory action of the phenylcarboxyl group of PCMB in competition with the substrate is thought to be involved in the inhibitory action of PCMB observed by Singer and Barron[7]. On the other hand, Frisell and Hellerman[9] reported that the inhibitory action of phenylmercuric acetate is not in competition with the substrate. From these results, it may be considered that the competitive inhibition of PCMB with the substrate may be attributed to the phenylcarboxyl group of the inhibitor.

As to the competition of PCMB with FAD, it may be attributed to its sulfhydryl-reactive group, since it was made clear that the phenylcarboxyl group does not take part in the competition with FAD and that the competition of PCMB with FAD was found to be of a single mechanism. Moreover, the competition of PCMB was attributed to its competition with the AMP part of FAD as mentioned before.

Thus, the conclusion deduced from the above considerations that the sulfhydryl group of D-amino acid oxidase apo-protein combines with the AMP part of FAD may solve the confusion[7–10] regarding the binding site of the sulfhydryl group of the apo-protein.

From these results, it should also be noticed that the binding between FAD and the apo-protein of D-amino acid oxidase occurs at least in two portions.

Furthermore, it was noticed that there is an important interaction between the binding sites of the apo-protein with FMS and AMS. The present results indicated that the attachment of AMS to the apo-protein increases the affinity of the apo-protein for FMS, and that the binding of FMS increases the affinity of the apo-protein for AMS. These positive interactions were also observed between the binding site of FMS and that of PCMB, and the binding site of AMS and that of PABA.

These demonstrations of positive interaction are of special interest in the study of the combination of the apo-protein with FAD, and consequently, of the mechanism of action of this enzyme.

REFERENCES

[1] K. Yagi, J. Okuda, T. Ozawa and K. Okada, *Science*, 124 (1956) 273.
[2] K. Yagi, J. Okuda, T. Ozawa and K. Okada, *Biochem. Z.*, 328 (1957) 492.
[3] K. Yagi, J. Okuda, T. Ozawa and K. Okada, *Biochim. Biophys. Acta*, 34 (1959) 372.
[4] K. Yagi, T. Ozawa and K. Okada, *Biochim. Biophys. Acta*, 35 (1959) 102.
[5] K. Yagi and T. Ozawa, *Biochim. Biophys. Acta*, 39 (1960) 304.
[6] H. Theorell and P. A. Nygaard, *Acta Chem. Scand.*, 8 (1954) 1649.
[7] T. P. Singer and E. S. G. Barron, *J. Biol. Chem.*, 157 (1945) 241.
[8] T. P. Singer, *J. Biol. Chem.*, 174 (1948) 11.
[9] W. R. Frisell and L. Hellerman, *J. Biol. Chem.*, 225 (1957) 53.
[10] H. Kubo, T. Yamano, M. Iwatsubo, H. Watari, T. Soyama, J. Shiraishi, S. Sawada, N. Kawashima, S. Mitani and K. Ito, *Bull. Soc. Chim. biol.*, 40 (1958) 431.
[11] K. Yagi and T. Ozawa, *Nature*, 184 (1959) 1227.
[12] E. Negelein and H. Brömel, *Biochem. Z.*, 300 (1939) 225.
[13] K. Yagi, Y. Matsuoka, S. Kuyama and M. Tada, *J. Biochem.*, 43 (1956) 93.
[14] N. Takahashi, K. Yagi and F. Egami, *J. Chem. Soc. Japan, Pure Chem. Sect.*, 78 (1957) 1287.
[15] F. Egami and N. Takahashi, *Bull. Chem. Soc. Japan*, 28 (1955) 666.

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THE ACTIVATION AND INCORPORATION OF AMINO ACIDS BY SUBCELLULAR FRACTIONS OF *BACILLUS MEGATERIUM* STRAIN M[*]

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SUMMARY

Cell fractions obtained by the lysis of protoplasts of *B. megaterium* strain M were analysed for protein, nucleic acid and the amino-acid-activating and -incorporating enzymes. The fraction including the cytoplasmic membranes contains an average of 17% of the cell protein, 12% of the cell RNA, and 37% of the cell DNA. The amino-acid-incorporating ability of this fraction is greater than that of any other cell fraction, and the amino acids are incorporated under conditions in which no amino-acid-dependent PP exchange with ATP can be demonstrated. Essentially all of the amino-acid-activating enzymes are associated with the supernatant of a 24,000 × *g* centrifugation. The incorporating activity of the "membrane fraction" is stimulated by soluble RNA from either *B. megaterium* or *E. coli*, a mixture of ribonucleoside diphosphates, and by fragmentation of "membranes" with either digitonin or perfluorooctanoate. Incorporation into a hydroxylamine-stable form is insensitive to the action of ribonuclease and is sometimes depressed by the presence of a complete amino acid mixture. The significance of these findings is discussed.

INTRODUCTION

In recent years, there has been an increasing amount of evidence to suggest a central metabolic role for the bacterial cytoplasmic membrane. Since more than 90% of the bacterial cytochromes, succinic dehydrogenase, and other oxidative enzymes

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Abbreviations used in this paper: ATP = adenosine triphosphate; ADP = adenosine diphosphate; GTP = guanosine triphosphate; PP = pyrophosphate; RNA = ribonucleic acid; DNA = deoxyribonucleic acid; RNAase = ribonuclease; S-RNA = soluble RNA; Tris = tris (hydroxymethyl)aminomethane; Medium D = 0.04 *M* Tris buffer pH 7.4 containing $2 \cdot 10^{-3}$ *M* $MgSO_4$; PFO = perfluoro-octanoate; TCA = trichloracetic acid; XDP = mixture containing equal amounts of each of the four ribonucleoside diphosphates; AA = equilibrated amino acid mixture containing L-amino acids in ratios corresponding to their occurrence in *E. coli* proteins; ala = alanine; arg = arginine; asp = aspartic; asp-NH_2 = asparagine; cys-Sh = cysteine; glu = glutamic; glu-NH_2 = glutamine; gly = glycine; his = histidine; ileu = isoleucine; leu = leucine; lys = lysine; meth = methionine; phe = phenylalanine; pro = proline; ser = serine; thr = threonine; try = tryptophan; tyr = tyrosine; val = valine.

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are found associated with the membranes of *Bacillus megaterium*[1, 2] and *Staphylococcus aureus*[3], it is apparent that membranes perform many of the enzymic functions of mammalian mitochondria[4].

In addition, there is substantial evidence that the bacterial cytoplasmic membrane is the principal site for protein biosynthesis, and therefore has much in common with the liver microsome[5].

When protoplasts of either *B. megaterium*[6] or *Escherichia coli*[7] are exposed to radioactive amino acids for short periods of time and then fractionated with respect to cell components, the specific activity of the membrane proteins is found to be greater than that of other cell proteins. Furthermore, experiments with isolated cell membranes of *B. megaterium* strain KM by BUTLER *et al.*[6] have shown that of all the components of a total lysate, the membrane fraction is the most active in incorporating amino acids into protein. Similar results have been reported for membranes and membrane fragments of *E. coli* (NISMAN[8]; SPIEGELMAN[7]; NISMAN AND HIRSCH[16]) membrane fragments of *Alcaligenes faecalis* and *Azotobacter vinelandii* (BELJANSKI AND OCHOA[9]), and the membrane-containing fraction of *Staphylococcus aureus* (GALE[10]).

Experiments with liver microsomal and supernatant systems[11, 12, 13] have greatly influenced the current ideas on protein synthesis. It is commonly thought that the following activation reaction is the first enzymic step in protein synthesis:

$$\text{ATP} + \text{amino acid} \rightleftarrows \text{adenyl-amino acid} + \text{PP}$$

the amino acid is then transferred to low molecular weight RNA[13, 14] and is then incorporated into protein in the presence of ribonucleoprotein particles and GTP[15]. The incorporation is strongly inhibited by the presence of ribonuclease. Results consistent with this scheme have been obtained with preparations from *E. coli* (SPIEGELMAN[7]; NISMAN[8] and NISMAN AND HIRSCH[16]). The washed membranes containing fraction incorporates amino acids, carries out an amino-acid-dependent PP exchange with ATP, and contains RNA. However, NISMAN[8] was unable to demonstrate an effect of GTP on incorporation.

Recently, both NISMAN AND FUKUHARA[17] and BELJANSKI AND OCHOA[9] have described bacterial amino-acid-incorporating systems, with properties different from those of the liver supernatant systems. Phenol-treated *E. coli* membrane fragments[17] and membrane fragments of *Alcaligenes faecalis*[9] incorporate amino acids, but do not catalyse an amino-acid-dependent PP exchange reaction with ATP. Nor are they able to form the complex S-RNA-AA. The activity of the *E. coli* membrane fragments is stimulated by the presence of RNA-ase[17].

The work reported in this paper was undertaken in an attempt to localize both the amino-acid-incorporating system and the amino-acid-activating systems in a lysozyme-sensitive organism, in which there would be no interference by the cell wall material. In addition, the cell fractions were analysed for protein and nucleic acid. The results show that the amino-acid-incorporating ability of the fraction containing the membranes of *B. megaterium* is greater than that of any other cell component, and that this activity is not inhibited by RNA-ase. The fraction including the membranes contains an average of 12% of the cell RNA, 37% of the cell DNA, and at most traces of amino-acid-activating enzymes.

MATERIALS AND METHODS

Preparation of enzyme fractions

We are indebted to Dr. IONESCO for a culture of *Bacillus megaterium* strain M[18]. Cells were grown with vigorous aeration at 30° on a medium consisting of 0.3% Bacto casitone, 0.3% Bacto tryptone, 0.2% Bacto peptone, 0.1% yeast extract, 0.5% K_2HPO_4, 0.05% $MgCl_2$, and 0.01% $FeSO_4 \cdot 7H_2O$ (pH adjusted to 7.2)*. Cells were harvested by centrifugation after growth for 12–18 h, washed once with 0.04 *M* Tris buffer pH 7.4 containing $2 \cdot 10^{-3}$ *M* $MgSO_4$ (Medium D), and were finally resuspended in Medium D containing 0.25 *M* sucrose.

For the preparation of protoplasts, crystalline lysozyme was added to a final concentration of 0.4 mg per ml. Although the lysozyme to cell ratio was kept constant, there was a considerable day-to-day variation in the speed of lysozyme action. Protoplast formation was essentially complete after incubation at room temperature for from 20 to 150 minutes. Protoplasts were collected by centrifugation at 13,000 *g* for 10 minutes and were resuspended in Medium D containing 0.25 *M* sucrose.

Protoplasts were lysed at 4° either by osmotic shock or by lysis in 0.25 *M* sucrose in the presence of digitonin[16, 37]. For osmotic lysis, enough Medium D was added to protoplasts suspended in hypertonic sucrose (0.25 *M*) to lower the sucrose concentration to 0.04 *M*. Lysis was complete after stirring for 5 minutes. The addition of digitonin (final concentration of 3–7 mg/ml) to protoplasts suspended in hypertonic sucrose resulted in complete lysis after 5–10 minutes of stirring. With either procedure, the $MgSO_4$ concentration of the total lysate was adjusted to 10^{-2} *M*.

All the steps involved in the fractionation of the total lysate were performed at 4°. The first low speed centrifugation (Fig. 1) separates the total lysate into a membrane (P_1)- and supernatant (S_1)-containing fractions. The ribosomes[19] (P_4) present in the S_1 fraction were obtained by centrifugation at 100,000 × *g*. The P_1 fraction was washed once and in some experiments, the resulting P_2 fraction was treated with either digitonin or the anionic detergent, perfluoro-octanoate (PFO)**. These compounds induce membrane fragmentation. The supernatant fractions (S_2, S_3, S_4, S_6) were precipitated with 2 volumes of ethanol at —20°. The resulting pellets were redissolved in Medium D and then assayed for enzymic activity.

Definition of the fractions obtained by differential centrifugation

Cell fractions obtained from osmotically shocked protoplasts by differential centrifugation can be defined essentially as follows:

(1) fraction (P_1) or (P_2) or "membranes" sedimented at 24,000 × *g* including the bulk of the cytoplasmic membranes and associated with a variable amount of DNA and RNA.

(2) fraction (S_1) which submitted to high speed centrifugation for 120 minutes or more, separates into two major components.

(S_4) corresponding to the soluble proteins and containing the SRNA

(P_4) containing the bulk of the cellular free ribosomes.

Measurement of amino acid incorporation

(a) *Incorporation into protein and nucleic acid fractions.* Cell fractions, incubated

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* Medium devised by NISMAN as a substitute for Pennassay medium.

** Produced by the Minnesota Mining and Manufacturing Co., St. Paul, Minn.

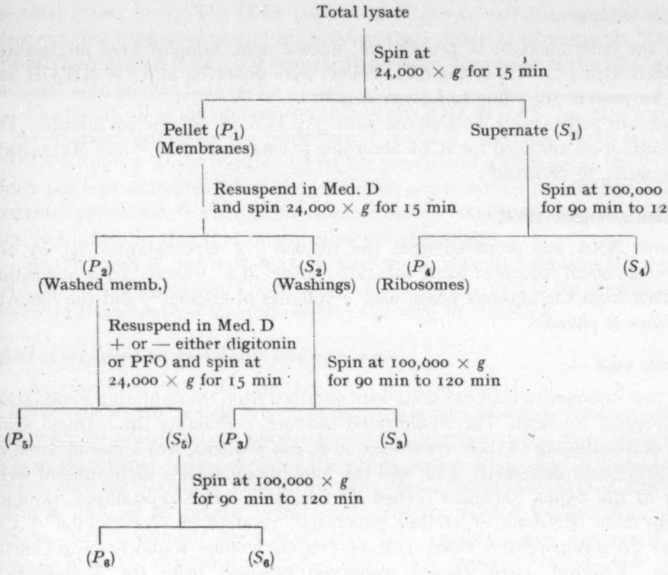
Total lysate
Spin at 24,000 × g for 15 min
Pellet (P_1) (Membranes)
Supernate (S_1)
Resuspend in Med. D and spin 24,000 × g for 15 min
Spin at 100,000 × g for 90 min to 120 min
(P_2) (Washed memb.)
(S_2) (Washings)
(P_4) (Ribosomes)
(S_4)
Resuspend in Med. D + or — either digitonin or PFO and spin at 24,000 × g for 15 min
Spin at 100,000 × g for 90 min to 120 min
(P_5)
(S_5)
(P_3)
(S_3)
Spin at 100,000 × g for 90 min to 120 min
(P_6)
(S_6)

Fig. 1. Centrifugal fractionation of total lysate.

in the presence of radioactive amino acids, were precipitated with 5% TCA in the cold and were allowed to flocculate for about one hour. Approximately 1 mg of protein carrier was added and each precipitate was washed 5 times with 5% TCA in the cold. Pellets were dissolved in 0.7 *N* NH_4OH and aliquots were dried on aluminium planchets for counting. These are referred to as "total counts".

(b) *Incorporation into protein*. The amino acid–RNA bond is hot acid-[14,17], alkali-[13,14] and hydroxylamine (NH_2OH)-[14,17] labile. In experiments with *E. coli*, NISMAN[20] found that the hot acid (5% TCA) stable count is equivalent to that obtained by treatment with NH_2OH (*M*) at pH 7.0.

To insure complete solubilization of the amino acids bound to RNA, the ammonium hydroxide solubilized pellets were neutralized, and the NH_2OH concentration was adjusted to 1 *M*. Samples were left at room temperature for a minimum of one hour, precipitated, washed with 5% TCA in the cold, and then dissolved in 0.7 *N* NH_4OH. Aliquots were plated and counted. These are referred to as "NH_2OH-stable" counts and presumably represent amino acid incorporated into protein.

All samples were counted at infinite thinness in a Nuclear gas flow counter.

Measurement of [^{32}P] PP incorporation into ATP

[^{32}P] PP exchange with ATP was measured according to the technique of BERG[21] as modified by NISMAN AND HIRSCH[16].

Analytical procedures

For the determination of protein and nucleic acid, samples were precipitated and washed with 5% TCA in the cold. Pellets were dissolved in 0.7 *N* NH_4OH and assayed for protein according to LOWRY *et al.*[22].

Duplicate pellets were hydrolysed with 5% TCA at 95° for 20 minutes. The supernatants were analysed for RNA according to CHARGAFF *et al.*[23] and MEJBAUM[38], DNA according to BURTON[24].

Preparation of soluble RNA

Soluble RNA was prepared from the 100,000 × *g* supernatants (S_4), by the addition of an equal volume of water-saturated phenol at 4°, followed by precipitation of the RNA from the aqueous phase with 2 volumes of ethanol[20], and the removal of the traces of phenol.

Compounds used

The non-radioactive L-amino acids were obtained from the California Foundation for Biochemical Research. The equilibrated mixture, containing the L-amino acids in ratios corresponding to their occurrence in *E. coli* proteins, was a modification of the one previously described[8]. ATP and the four ribonucleoside diphosphates were products of the Sigma Chemical Company. Lysozyme twice crystallized, product of Armour & Co. RNA-ase crystalline pancreatic, product of Worthington & Co. Digitonine (crystalline) Merck, Darmstadt. DL-[^{35}S]methionine, Radiochemical Centre, Amersham, England. L-[^{14}C]alanine uniformyl labelled, from the Radiocarbon Laboratory of the Pasteur Institute (Drs. Aubert and Milhaud) Paris. [^{32}P]PP prepared by alkaline fusion at 400° for 60 min and separated on Dowex 1-Cl according to BERG[21].

RESULTS

Distribution of protein and nucleic acid

Cell fractions obtained from osmotically shocked protoplasts were analysed for RNA, DNA, and protein. The results (Table I) show that the fraction including the washed membranes (P_2) of *B. megaterium* contains an average of 17% of the cell protein, 12% of the cell RNA, and 37% of the cell DNA. This fraction has the highest DNA/protein ratio (0.12) and an RNA/protein ratio (0.11) lower than that of either

TABLE I

DISTRIBUTION OF PROTEIN AND NUCLEIC ACID IN ISOLATED CELL FRACTIONS

The results represent an average of at least two experiments and are expressed as % of total lysate (*TL*), and ratio of RNA and DNA to protein.

Fraction	% *Protein of TL*	% *RNA of TL*	% *DNA of TL*	$\frac{RNA}{Protein}$	$\frac{DNA}{Protein}$
TL	100	100	100	0.15	0.051
S_1	74.0	75.8	58.6	0.15	0.042
P_2 (washed membranes)	17.2	12.4	37.4	0.11	0.123
S_2 (washings)	1.6	2.9	4.5	0.24	0.102
P_4 (ribosomes)	14.5	54.4	13.8	0.59	0.048
S_4	61.9	12.1	14.3	0.031	0.013

the total lysate or S_1. The DNA content of (P_2) was variable, with DNA/protein ratios ranging from 0.09 to 0.17 obtained in three different experiments. The RNA/protein ratios varied from 0.071 to 0.12. In all cases, recoveries in the (P_2), S_1 and S_2 fractions were greater than 90%.

Essentially all of the protein and 88% of the RNA of the S_1 fraction is recovered in the fraction containing the ribosomes (P_4) and S_4 fractions. Since the recovery of DNA is only 48%, the results suggest the presence of a DNAase in the S_1 fraction, which becomes activated when the ribosomes are removed by centrifugation. (P_4) contains approximately 72% of the RNA of the S_1 fraction and has an RNA/protein ratio (0.59) greater that that of any other cell component.

As the result of one washing with Medium D, there is a preferential removal of RNA from the (P_2) fraction. The washings (S_2) contain 9–10% of the protein and DNA present in the unwashed membranes (P_1) and 20% of the RNA.

Effect of digitonin on protoplasts and membranes

As is shown in Fig. 2, the addition of 1.0 mg of digitonin to a protoplast suspension in hypertonic sucrose results in a rapid decrease in optical density (curve 3). Observations with the phase contrast microscope when the optical density had fallen to about 35% of its original value, revealed the presence of granules and cytoplasmic membranes, but the complete absence of protoplasts. When the optical density had fallen to 20% of its original value, only granules were observed. Thus, digitonin induces both protoplast lysis and membrane fragmentation. The speed of lysis depends upon the digitonin/protoplast ratio, as is shown by the increase in rate of decline when an additional 1.0 mg of digitonin is added (curve 3). The presence of 0.1 mg of digitonin (curve 2) induces little protoplast lysis. The control protoplast suspension (curve 1) increases in optical density during the incubation period, owing to protoplast contraction.

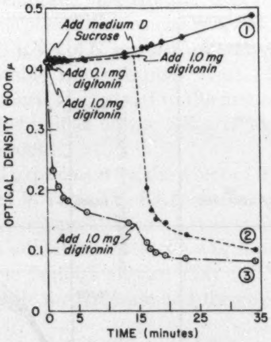

Fig. 2. Digitonin-induced lysis of protoplasts in hypertonic sucrose. Each cuvette contained 3.0 ml of a washed protoplast suspension in medium D sucrose (0.25 *M* sucrose). Digitonin was dissolved in medium D sucrose and all additions to cuvettes were made in 0.01 ml quantities. At zero time medium D sucrose was added to cuvette 1, 0.1 mg digitonin to cuvette 2, and 1.0 mg digitonin to cuvette 3. These additions were repeated after 14½ min, except that 1.0 mg digitonin was added to cuvette 2. Temp. = 24°. The optical density was read at 600 mμ against a water blank.

The exposure of washed "membranes" (P_2) to either digitonin or perfluorooctanoate at 4° also results in fragmentation. Although neither membranes nor membrane fragments can be detected with the phase contrast microscope, some of the fragments are large enough to be sedimentable at 24,000 × *g*. As much as 30 % of the P_2 protein may be recovered as large fragments.

Incorporation of labelled amino acids

Membrane fragments from protoplasts lysed with digitonin at 4° were tested for their ability to incorporate radioactive amino acids. The results (Fig. 3) show that both the total count and the NH_2OH-stable count increase over a period of 2 hours. The NH_2OH count varies from 50–60% of the total count. Both types of incorporation are stimulated by ATP, by a mixture of all four ribonucleoside diphosphates or triphosphates, and by a preparation of S-RNA from *E. coli*. During the first 90 minutes, S-RNA is not as effective as a mixture of ribonucleoside diphosphates in stimulating incorporation. This is consistent with the finding of, at most, traces of amino-acid-activating enzymes in "membranes" and suggests that this stimulation is due to nucleotides derived from S-RNA.

The major cell fractions were tested for their ability to incorporate amino acids. The results (Table II) show that of all components of a total lysate, the "membranes" are the most active in incorporating amino acids into a NH_2OH-stable form. While the activity of the S_1 fraction is only slightly lower than that of the total lysate, the ribosome (P_4) and S_4 fractions have the poorest incorporating abilities. The NH_2OH-labile counts associated with the "membrane" are lower than those associated

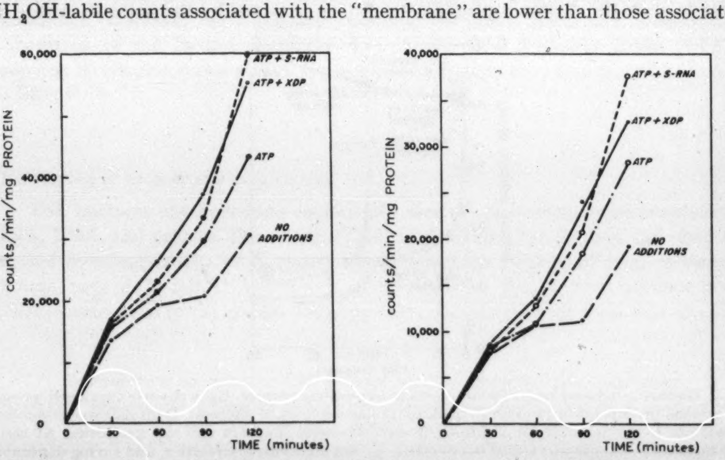

Fig. 3. Incorporation of [^{35}S]methionine and [^{14}C]alanine by membrane fragments. 3A = total incorporation; 3B = NH_2OH stable incorporation. The membrane fragments were obtained by digitonin lysis of protoplasts and were used without washing. The reaction mixtures were incubated aerobically with shaking at 30°. Each flask contained the following constituents per ml: 540 μg membrane protein, 55 μmoles Tris buffer pH 7.3, 1.1 μmoles $MgSO_4$, 0.1 μmoles $MnCl_2$, 260 μg equilibrated amino acid mixture, 66 mμmoles L-[^{35}S]methionine of 7,500 counts/min/mμmole, and 10 mμmoles L-[^{14}C]alanine of 3,800 counts/min/mμmole. Additions per ml: 0.55 μmoles ATP, 44.4 μg XDP mixture containing 11.1 μg of each of the four ribonucleoside diphosphates, and 222 μg S-RNA from *E. coli*.

TABLE II

LOCALIZATION OF THE AMINO-ACID-INCORPORATING SYSTEM

The cell fractions were obtained from osmotically lysed protoplasts. The membrane fraction was washed once. The reaction mixtures were incubated aerobically at 30°. Each mixture contained the following constituents per ml: 35 μmoles Tris buffer pH 7.3, 1.0 μmole $MgSO_4$, 1.0 μmole $MnCl_2$, 0.52 μmoles ATP, 41.6 μg XDP mixture containing 10.4 μg of each of the four ribonucleoside diphosphates, 122 μg equilibrated amino acid mixture, 220 mμmoles L-[^{35}S]methionine of 750 counts/min/mμmole, and the following amounts of protein: 207 μg *TL*, 202 μg S_1, 144 μg P_2, 350 μg P_4, and 680 μg S_4. The results are expressed as the change in specific activity over a period of 80 min.

Fraction	*CPM/mg Protein*		
	Total counts	*NH_2OH-stable count*	*Counts liberated by NH_2OH*
TL (total lysate)	5710	1867	3843
S_1	5500	1703	3797
P_2 (membranes)	3720	2780	940
P_4 (ribosomes)	2900	1010	1890
S_4	2656	716	1940

with either the total lysate or S_1 fractions. This is consistent with the lower RNA content of "membranes".

(P_2) fraction obtained by osmotic lysis of protoplasts was exposed to either digitonin or PFO at 4°. The resulting large fragments were recovered by centrifugation at 24,000 × *g* and the incorporating ability of these fragments was compared with that of a twice-washed "membrane" preparation. The results (Table III) show that both types of fragments incorporate amino acids more rapidly than the "membrane" control (P_5A). In all cases, S-RNA of *B. megaterium* stimulates both the total incorporation and the NH_2OH stable incorporation, while the equilibrated amino acid mixture has an inhibitory effect. In contrast to the first experiment on incorporation (Fig. 3), ATP has only a slight effect on the NH_2OH stable count, and often an inhibitory effect on the total count.

The results of a typical experiment on the effect of RNAase on the incorporating ability of (P_2) are presented in Table IV. RNA-ase, added at zero time, has a slight inhibitory effect on the total incorporation and has essentially no effect on the NH_2OH stable count. Soluble RNA stimulates both types of incorporation, especially in the presence of RNA-ase. These findings suggest that S-RNA stimulates by virtue of its ability to act as a substrate for RNA-ase and thereby furnishes nucleotides.

Amino acid activation

Cell fractions, obtained by osmotic lysis of protoplasts, were tested for their ability to catalyse an amino-acid-dependent PP exchange with ATP. The results of 2 experiments are summarized in Table V. To facilitate the assay of many cell fractions, mixtures of amino acids were used. Although the fractions were not dialysed to remove free amino acids, stimulations in PP exchange were observed upon the addition of amino acid mixtures. This was true even of fractions expected to contain the highest endogenous amino acid levels (*TL*, S_1).

It is clear that most of the amino-acid-stimulated exchange activity of the total lysate can be accounted for by the S_1 fraction. In fact, in the presence of the

TABLE III

EFFECT OF DIGITONIN AND PERFLUORO-OCTANOATE TREATMENT ON INCORPORATING ABILITY OF MEMBRANES

Cell membranes, obtained from osmotically lysed protoplasts, were washed once. The membranes (P_2) were then incubated with either medium D alone, or with medium D containing digitonin at a final concentration of 3 mg/ml, or PFO at a final concentration of 9 mg/ml. After 20 min at 4°, the preparations were centrifuged at 24,000 × *g* for 15 min, and the resulting pellets (P_5) tested for ability to incorporate amino acids. The reaction mixtures were incubated aerobically with shaking at 30°. Each flask contained the following constituents per ml: 65 μmoles Tris buffer pH 7.3, 1.5 μmoles $MgSO_1$, 1.5 μmoles $MnCl_2$, and 220 mμmoles L-[^{35}S]methionine of 750 counts/min/mμmole. Additions per ml: 0.62 μmoles ATP, 585 μg equilibrated amino acid mixture (without methionine), 74 μg S-RNA from *B. megaterium*, and the following amounts of protein: 231 μg P_5A (Medium D-treated), 234 μg P_5B (digitonin-treated), and 204 μg P_5C (PFO-treated).

Additions	*Treatment*	*Fraction*	*Time (min)*	*Counts/min/mg Protein*	
				Total counts	*NH_2OH-stable count*
—	Medium D	P_5A	40	4500	2090
			80	7000	2500
ATP		P_5A	40	3480	1870
			80	5050	3030
ATP, S-RNA		P_5A	40	4470	2840
			80	5910	2620
ATP, AA		P_5A	40	2960	1660
			80	3540	2010
—	Digitonin	P_5B	40	6200	2770
			80	6770	3810
ATP		P_5B	40	5640	2770
			80	7340	3300
ATP, S-RNA		P_5B	40	7110	3680
			80	9170	4420
ATP, AA		P_5B	40	4310	2830
			80	4440	2830
—	PFO	P_5C	40	6440	2450
			80	6170	3530
ATP		P_5C	40	4940	2550
			80	6450	3730
ATP, S-RNA		P_5C	40	6380	3870
			80	7160	5100
ATP, AA		P_5C	40	4710	2160
			80	5100	2800

most active amino acid mixtures, the specific activity of the S_1 fraction is greater than that of the total lysate. The washed membranes (P_2) contained small amounts of activity in Expt. 4 and were completely inactive in Expt. 5. In the latter experiment, the addition of amino acid mixtures inhibited the endogenous PP exchange activity

TABLE IV

EFFECT OF RNAASE ON INCORPORATING ABILITY OF MEMBRANES

Cell membranes, obtained from osmotically lysed protoplasts, were washed once. The reaction mixtures were incubated aerobically with shaking at 30°. Each flask contained the following constituents per ml: 37 μmoles Tris buffer pH 7.3, 1.0 μmole $MgSO_4$, 1.0 μmole $MnCl_2$, 334 μg equilibrated amino acid mixture, 117 mμmoles L-[^{35}S]methionine of 2,010 counts/min/mμmole, and 161 μg membrane protein. Additions per ml: 143 μg RNAase, and 60 μg S-RNA (mixtures of equal parts from *E. coli* and *B. megaterium*).

Additions	*Time (min)*	*Counts/min/mg Protein*	
		Total counts	*NH_2OH-stable count*
—	40	2735	1380
	80	2670	1520
RNAase	40	2220	1370
	80	2550	1560
S-RNA	40	2920	1560
	80	3600	1930
RNAase, S-RNA	40	2980	920
	80	7770	3260

of the P_2 fraction. Results similar to those of Expt. 5 were obtained in a third experiment. Activating enzymes are readily removed from "membranes" by washing, as is shown by the finding of low levels of activity in the S_2 fraction and none in the P_2 fraction (Expt. 5). This suggests that the "membranes" of Expt. 4 were improperly washed and were, therefore, contaminated with the S_1 fraction.

About 80% of the activity of the S_1 fraction is recovered in the ribosome (P_4) and S_4 fractions, with S_4 somewhat more active than P_4. The low levels of amino-acid-stimulated activity associated with P_2 in Expt. 4 are lost after fragmentation of the membranes with digitonin. The large fragments (P_5B) have a slightly higher level of endogenous PP exchange than the P_2 fraction. However, values below the endogenous level are obtained when amino acid mixtures are added to P_5B.

DISCUSSION

The fraction including the membrane (P_2) of *B. megaterium* strain M contains an average of 12% of the cell RNA and 37% of the cell DNA. BUTLER *et al.*[6] reported that the membranes of *B. megaterium* strain KM contain 13% of the cell RNA and 54% of the DNA (data calculated from their paper). NISMAN AND FUKUHARA[37] reported that the fraction containing the membrane fragments in *E. coli* contains 80–85% of the total cell DNA. However, there have been conflicting reports as to the presence of nucleic acid in bacterial membranes. VENNES AND GERHARDT[25] found RNA, but no DNA, in the membranes of strain KM, while GILBY *et al.*[27] could find no more than traces of nucleic acid in the membranes of *Micrococcus lysodeikticus*. WEIBULL *et al.*[26] originally reported that nucleic acids were essentially absent from the membranes of strain M and subsequently reported that membranes of this strain contain no more than 5% of the cell RNA[2]. In addition, there have been reports

TABLE V

LOCALIZATION OF THE SYSTEM CATALYZING THE AMINO-ACID-DEPENDENT EXCHANGE OF PP WITH ATP

The cell fractions were obtained from osmotically lysed protoplasts. Expt. 4. An aliquot of washed membranes (P_2) was incubated with digitonin (final concentration of 4.5 mg/ml) at 4° for 15 min and then centrifuged at 24,000 × g for 15 min. The supernatant (S_5B) was then centrifuged at 100,000 × g for 90 min. The pellets (P_5B and P_6B) were resuspended in medium D, while the protein of S_6B was precipitated with 1 vol. of ethanol and then suspended in medium D. Each reaction mixture contained the following constituents per ml: 100 µg protein of each fraction tested, 100 µmoles Tris buffer pH 8.0, 5.0 µmoles $MgSO_4$, 10.0 µmoles KF, 2.0 µmoles ATP, and 1.75 µmoles sodium pyrophosphate of 27,500 counts/min/µmole. Mixtures of L-amino acids were added to give a final concentration of 2.0 µmoles of each amino acid per ml. Reaction mixtures were incubated for 15 min at 30° and the reaction was stopped by adding cold TCA. Expt. 5. Reaction mixtures contained the same constituents as those used in Expt. 4, except that the sodium pyrophosphate concentration was increased to 2.0 µmoles/ml (58,900 counts/min/µmole). Mixtures were incubated for 15 min at 30° and the reaction was stopped by adding cold TCA.

	mM moles [^{32}P]PP incorporated into ATP per mg protein											
	Expt. 4						*Expt. 5*					
	Amino acids added						*Amino acids added*					
Fraction	*none*	*ala* *arg* *glu* *glu–NH$_2$* *gly*	*thr* *asp* *asp–NH$_2$* *ser* *lys*	*cys–SH* *meth*	*val* *leu* *ileu*	*pro* *phe* *try* *tyr* *his*	*none*	*ala* *arg* *glu* *glu–NH$_2$* *gly*	*thr* *asp* *asp–NH$_2$* *ser* *lys*	*cys–SH* *meth*	*val* *leu* *ileu*	*pro* *phe* *try* *tyr* *his*
TL	364	378	320	524	712	528	237	356	485	700	1470	509
-Endog		14	—	160	348	164		119	248	463	1233	272
S_1	375	295	346	618	1465	488	142	314	440	875	1720	505
-Endog		—	—	243	1090	113		172	298	733	1578	363
P_2	269	306	287	298	324	331	95	20	10	20	65	27
-Endog		37	18	29	55	62		—	—	—	—	—
S_2							41	34	44	88	163	54
-Endog								—	3	47	122	53
P_4	135	218	156	269	222	182						
-Endog		83	21	134	87	47						
S_4	204	207	193	389	582	197						
-Endog		3	—	185	378	—						
P_5B	298	84	76	171	167	164						
-Endog		—	—	—	—	—						
P_6B	95	58	134	98	69	91						
-Endog		—	39	3	—	—						
S_6B	182	87	149	142	142	80						
-Endog		—	—	—	—	—						

of the association of nuclear bodies with the membrane fraction of strain KM[28,29]. These bodies contain RNA, DNA, and protein in a ratio of 1:1:3. At present, it is difficult to explain the contradictory findings as to the presence of nucleic acid in membranes or to determine to what extent the finding of DNA in "membranes" indicates contamination with nuclear bodies.

The endogenous PP exchange activity of the membrane fraction is lower than that of either the total lysate or the S_1 fraction. This endogenous activity could be due to the presence of amino acids, fatty acids[14,30], inorganic acids[31,32], or to the residual PP exchange activity found in dialysed extracts[33]. However, the addition of amino acid mixtures to the total lysate and S_1 fraction does result in an increased rate of PP exchange. These fractions would be expected to contain the highest endogenous amino acid levels. No such stimulation was observed in two experiments with (P_2) and only a slight stimulation was observed in a third experiment. This low level of activity was lost after fragmentation of the membranes with digitonin. Therefore, "membranes" capable of incorporating amino acids into a NH_2OH-stable form do not necessarily contain amino-acid-activating enzymes, as measured by PP exchange with ATP. They may contain small amounts of these enzymes if they are improperly washed. Experiments are now in progress to determine whether or not the activating enzymes have any effect on the amino acid incorporation by "membranes".

Although the S_1 fraction has appreciable amino-acid-incorporating ability, it is clear that the fraction containing the membranes is the most active component of the total lysate. The activity of the "membranes" is stimulated by S-RNA from either *B. megaterium* or *E. coli*, a mixture of all four ribonucleoside diphosphates, or by fragmentation with either digitonin or PFO. The stimulation in both the total and NH_2OH stable incorporation as the result of digitonin fragmentation, suggests that amino acid activation plays no role in either type of incorporation. "Membranes" obtained by osmotic lysis of protoplasts show a variable response to ATP. When "washed membranes" (P_2) are incubated with ATP, there is a 7% increase in the NH_2OH stable count and a 25% decrease in the total count (Table III). In an experiment with membranes from digitonin lysed protoplasts (Fig. 3), ATP induced a 50–60% stimulation in both types of incorporation. However, the membranes of the latter experiment were not washed.

In experiments with "membranes" the NH_2OH-stable count is usually 50–60% of the total count. The amino acids solubilized by NH_2OH treatment at pH 7.0 could be bound to ribonucleoprotein or to something analogous to the lipid-soluble amino acid complex of hen oviduct[34]. Since the presence of RNA-ase causes only a small decrease (15%) in the total count (Table IV), little of the amino acid can be linked to S-RNA. Also, this linkage would presumably occur via a mechanism not involving amino acid activation, as measured by PP exchange with ATP, or by subsequent formation of S-RNA-AA.

The incorporation of [^{35}S]methionine by "membranes" is depressed by the presence of a complete amino acid mixture. There is a decrease in both the total, and the NH_2OH-stable incorporation. The incorporation by *E. coli* membranes[7,8,16] is stimulated by the presence of all the other amino acids, while the complete amino acid mixture is without effect on the incorporation by rat liver supernatant[15]. Findings of inhibition are not too surprising, in view of recent evidence for the variable amino acid composition of bacterial proteins, in the presence of either naturally occurring amino acids or analogues[35].

The amino-acid-incorporating activity of the *B. megaterium* membrane is insensitive to RNA-ase, while the activity of phenol-treated *E. coli* membrane fragments is stimulated by RNA-ase[17]. Both systems, like *A. faecalis* membrane fragments[9] and PFO-disrupted rat liver microsomes[36], incorporate amino acids under conditions in which amino-acid-activating enzymes cannot be demonstrated.

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REFERENCES

[1] R. STORCK AND J. T. WACHSMAN, *J. Bacteriol.*, 73 (1957) 784.
[2] C. WEIBULL, H. BECKMAN AND L. BERGSTRÖM, *J. Gen. Microbiol.*, 20 (1959) 519.
[3] P. MITCHELL AND J. MOYLE, *Biochem. J.*, 64 (1956) 12 P.
[4] W. C. SCHNEIDER AND G. H. HOGEBOOM, *Ann. Rev. Biochem.*, 25 (1956) 201.
[5] J. W. LITTLEFIELD, E. B. KELLER, J. GROSS AND P. C. ZAMECNIK, *J. Biol. Chem.*, 217 (1958) 111.
[6] J. A. V. BUTLER, A. R. CRATHORN AND G. D. HUNTER, *Biochem. J.*, 69 (1958) 544.
[7] S. SPIEGELMAN, *Recent Progress in Microbiology*, Symposia held at VII Intern. Congr. for Microbiology (1958) 81.
[8] B. NISMAN, *Biochim. Biophys. Acta*, 32 (1959) 18.
[9] M. BELJANSKI AND S. OCHOA, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 494.
[10] E. F. GALE, *Symposium on Microbial Metabolism, Rome 1953*, p. 109.
[11] M. B. HOAGLAND, P. C. ZAMECNIK AND M. L. STEPHENSON, *Biochim. Biophys. Acta*, 24 (1957) 215.
[12] P. C. ZAMECNIK, M. L. STEPHENSON AND L. I. HECHT, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 73.
[13] M. B. HOAGLAND AND P. C. ZAMECNIK, *Federation Proc.*, 16 (1957) 197.
[14] P. BERG AND J. OFENGAND, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 78.
[15] E. B. KELLER AND P. C. ZAMECNIK, *J. Biol. Chem.*, 221 (1956) 45.
[16] B. NISMAN AND M. L. HIRSCH, *Ann. Inst. Pasteur*, 95 (1958) 615.
[17] B. NISMAN AND H. FUKUHARA, *Compt. rend.*, 248 (1959) 1438.
[18] J. TOMCSIK AND S. GUEX-HOLZER, *J. Gen. Microbiol.*, 10 (1954) 97.
[19] E. T. BOLTON, R. J. BRITTEN, D. B. COWIE AND R. B. ROBERTS, *Carnegie Institution of Washington Annual Report (1927–1958)*, p. 127.
[20] B. NISMAN, unpublished results.
[21] P. BERG, *J. Biol. Chem.*, 222 (1956) 1025.
[22] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[23] E. CHARGAFF, R. LIPCHITZ, C. GREEN AND M. E. HODES, *J. Biol. Chem.*, 192 (1952) 223.
[24] K. BURTON, *Biochem. J.*, 62 (1956) 315.
[25] J. W. VENNES AND P. GERHARDT, *Science*, 124 (1956) 535.
[26] C. WEIBULL AND L. BERGSTRÖM, *Biochim. Biophys. Acta*, 30 (1958) 340.
[27] A. R. GILBY, A. V. FEW AND K. MCQUILLEN, *Biochim. Biophys. Acta*, 29 (1958) 21.
[28] S. SPIEGELMAN, A. I. ARONSON AND P. C. FITZ-JAMES, *J. Bacteriol.*, 75 (1958) 102.
[29] P. C. FITZ-JAMES, *J. Bacteriol.*, 75 (1958) 369.
[30] W. JENCKS AND F. LIPMANN, *J. Biol. Chem.*, 225 (1957) 207.
[31] R. BANDURSKI, L. WILSON AND C. SQUIRES, *J. Am. Chem. Soc.*, 78 (1956) 6408.
[32] P. ROBBINS AND F. LIPMANN, *J. Am. Chem. Soc.*, 78 (1956) 6409.
[33] G. NOVELLI, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 86.
[34] R. HENDLER, *J. Biol. Chem.*, 234 (1959) 1466.
[35] D. COWIE AND G. COHEN, *Biochim. Biophys. Acta*, 26 (1959) 252.
[36] P. COHN, *Biochim. Biophys. Acta*, 33 (1959) 284.
[37] B. NISMAN AND H. FUKUHARA, *Compt. rend.*, 248 (1959).
[38] W. MEJBAUM, *Z. physiol. Chem., Hoppe Seyler's*, 258 (1939) 117.

Biochim. Biophys. Acta, 42 (1960) 388–400

METABOLIC POOLS AND THE UTILIZATION OF AMINO ACID ANALOGS FOR PROTEIN SYNTHESIS

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SUMMARY

Studies of the kinetics of analog and amino acid incorporation into metabolic pools and proteins of yeast and *E. coli* have shown that at least two processes are involved in the selection of the natural amino acid in preference to the analog. These selection steps occur in the formation of the metabolic pools, the first occurring in the concentrating system of the cell, which accumulates exogenous amino acids or analogs into the cell at levels exceeding external concentrations. In the yeast, the final distinction between analog and amino acid occurs at the time of entry into a second metabolic pool (internal pool). In this pool conversion of amino acids to the required end products occurs. Once incorporated into this pool no further selection occurs; the ratio of analog to amino acid in this pool and in protein is the same.

INTRODUCTION

The demonstration that amino acid analogs could be incorporated into bacterial proteins[1–9] raised many questions concerning (a) the nature of the protein synthesized and (b) the actual mechanism of analog incorporation. It has been shown that the substitution of the analogs norleucine or selenomethionine for methionine in the proteins of *E. coli* did not result in the synthesis of radically different molecular species, but that the macromolecules formed had physicochemical properties similar to the proteins normally synthesized[4,10]. Furthermore, each methionine site in the proteins seemed to have the same susceptibility for analog substitution, that is, norleucine[10] (and selenomethionine[4]) replaced methionine *in the same proportion* in all of the proteins examined. Recently it has been shown that the substitution of ethionine for methionine in a single protein (α-amylase) was the same for each individual methionine site[11].

As has been pointed out by YOSHIDA AND YAMASAKI[11], no evidence has been presented for the mechanism of analog incorporation into protein. It is evident that the bacterial cell possesses certain mechanisms for selecting the natural amino acid and rejecting, at least partially, the amino acid analog. With norleucine a large environmental pressure was required in order to effect a relatively small change in protein composition; a ratio in the medium of norleucine to methionine of 100 resulted in only a 40 % replacement of methionine by the analog[10]. Studies of the kinetics of analog incorporation into the metabolic pools and proteins of yeast and *E. coli* were made to determine where these selections among amino acids and their analogs occurred.

PROCEDURES

Wild type *E. coli* ML 30 and the yeast, *Candida utilis* were used in these experiments. Both types of cells were cultured in vigorously aerated C medium* with maltose as the carbon source for *E. coli* and fructose employed with *Candida utilis*.

DL-[3-^{14}C]-*p*-fluorophenylalanine was obtained from the Volk Radiochemical Company, Chicago, Illinois (79.2 mg/mC). Uniformly-labeled L-[^{14}C]phenylalanine was obtained from Nuclear-Chicago Corporation, Chicago, Illinois (16 mg/mC). These materials were always added directly to exponentially-growing cultures of cells with appropriate carrier compound to bring the concentration to the desired levels.

Culture samples of *E. coli* were withdrawn at various times and the cells removed by centrifugation. The cell pellet was washed by resuspending in 40 ml C medium and centrifuged. The resulting pellet was resuspended in 5 ml of 5 % cold trichloroacetic acid (TCA) and an appropriate aliquot measured to determine the total radioactivity taken up by the cells. The remaining suspension was centrifuged and the radioactivity of the TCA-soluble fraction (containing the metabolic pool) and of the TCA-insoluble fraction (containing the proteins) were measured to determine the distribution of radioactivity between pool and protein.

The extracting process used for *Candida utilis* was the same as that given above, except where it was desired to separate the two metabolic pools (see below) found in the yeast[15]. After washing in C medium the concentrating pool was extracted by resuspending the pellet of cells in 20 ml cold distilled water for 20 min.

An appropriate aliquot of this suspension was measured for the total radioactivity taken up by the cells. The remaining suspension was centrifuged to separate the water soluble fraction containing the expandable pool material. The pellet was treated with 5 % TCA and the distribution of tracer between the internal pool and protein was determined from measurement of radioactivity in the TCA soluble and insoluble fractions respectively.

RESULTS

E. coli

Kinetics of amino acid analog incorporation: The kinetics of incorporation and utilization of amino acid analogs are in many respects similar to those observed with the natural amino acids[12–16]. For example, at low external concentrations the analogs can be accumulated in the metabolic pool of the cell up to levels exceeding their external concentrations. This accumulation is rapid and precedes the appearance of the analog in the protein. Fig. 1 shows the time course of incorporation of tracer quantities of DL-[3-^{14}C]-*p*-fluorophenylalanine (*p*FPhe) into the pool and protein**.

---

* C Medium: 2 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 3 g NaCl, 0.01 g Mg as $MgCl_2$, 0.026 g S as Na_2SO_4, 100 ml 10 % maltose or fructose, and 900 ml distilled H_2O.

** *p*-fluorophenylalanine has been shown by MUNIER AND COHEN[9] to replace only phenylalanine in the proteins of *E. coli*.

The limited exogenous supply of the analog is soon exhausted and incorporation into protein rapidly depletes the analog in the pool.

At higher external concentrations of analog a proportionally larger quantity of accumulated material is observed in the cells. Fig. 2 (upper curve) shows the quantity of analog contained in the cold TCA-soluble fraction as a function of external concentration. Two processes appear to be involved in the uptake of *p*FPhe. One, a process concentrating analog in the cell in excess of the external concentration, appears to saturate at low concentrations. At exogenous levels exceeding 0.03 mg *p*FPhe/ml medium a second process of incorporation is observed. The quantity of material taken into the pool through this second process is directly proportional to the external concentration (dashed curve, Fig. 2). Pool accumulation to levels exceeding the external concentrations does not occur through this process. Incorporation of the analog into protein, however, as shown in Table I, depends directly upon the total quantity of pool material available, regardless of the mechanisms of uptake.

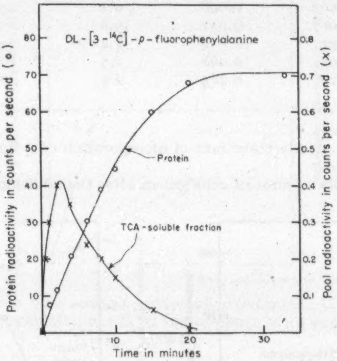

Fig. 1. Kinetics of uptake of tracer quantities of DL-[3-^{14}C] *p*-fluorophenylalanine (0.002 mg/ml medium) into pool and protein of *E. coli*.

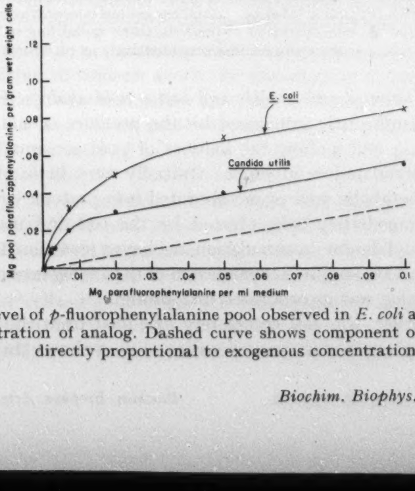

Fig. 2. Maximum level of *p*-fluorophenylalanine pool observed in *E. coli* and *C. utilis* as a function of external concentration of analog. Dashed curve shows component of accumulation which is directly proportional to exogenous concentration.

TABLE I

DISTRIBUTION OF PARAFLUOROPHENYLALANINE IN *Escherichia coli*

Medium *mg pFPhe/ml* *(at t = 0)*	*Pool** *mg pFPhe/g* *wet weight cells*	*Protein*** *mg pFPhe/Δ g* *wet weight cells*	*Protein/pool****
0.002	0.008	0.2	25
0.007	0.032	0.8	25
0.02	0.050	1.2	24
0.05	0.068	1.5	22
0.10	0.085	2.2	28

* Maximum level observed.

** Value calculated from steady state rate of incorporation of *p*-fluorophenylalanine per unit quantity of newly formed cells.

*** Δ gram wet weight cells = mass of cells grown after the addition of the tracer material.

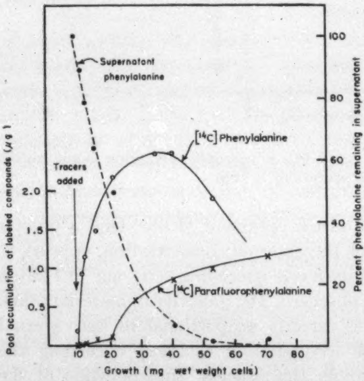

Fig. 3. ○, accumulation of L-phenylalanine; × DL-*p*-fluorophenylalanine in the pool of *E. coli*. An exponentially growing culture of cells was divided. Culture 1 (○) was supplied with L-[^{14}C]-phenylalanine and an equimolar quantity of DL-[^{12}C] *p*-fluorophenylalanine. Culture 2 (×) was treated identically, but with reciprocal labeling. Initial exogenous concentration of each compound was 0.0067 mg/ml medium. ●, concentration of phenylalanine remaining in medium. Growth of the two cultures was identical.

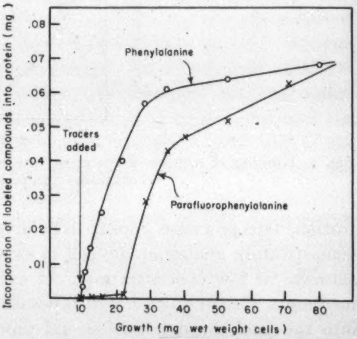

Fig. 4. ○, incorporation of L-phenylalanine; ×, incorporation of DL-*p*-fluorophenylalanine into proteins of *E. coli*. Conditions as described in Fig. 3.

Competitive utilization of amino acids and amino acid analogs: Kinetics of analog incorporation were significantly influenced by the presence of natural amino acids in the medium. Figs. 3 and 4 show the kinetics of pool accumulation and protein incorporation of phenylalanine and *p*FPhe. Initially very little of the analog was accumulated in the metabolic pool or incorporated into protein. On the other hand, phenylalanine was immediately concentrated by the cell and utilized for protein synthesis. When phenylalanine accumulation no longer continued at the maximal rate, due to depletion in the supply, accumulation of the analog into the pool occurred. Subsequently, the analog was incorporated into protein.

The analog, however, was not completely excluded from the cell, even during these early stages of phenylalanine incorporation. Fig. 5 shows the relative rates of protein incorporation of DL-[^{14}C]-p-fluorophenylalanine in the absence or presence of equimolar quantities of L-phenylalanine. The rate of protein incorporation of pFPhe was reduced by a factor of 100 when phenylalanine was present.

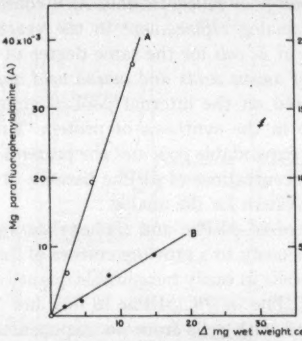

Fig. 5. Protein incorporation by *E. coli* of p-fluorophenylalanine. A, supplied in the absence of an equimolar quantity of phenylalanine; B, in the presence of an equimolar quantity of phenylalanine.

Candida utilis

Kinetics of amino acid analog incorporation: Similar experiments have been done with the yeast, *C. utilis*. In this organism *two* functionally distinct metabolic pools of amino acids have been found[15]. The first accumulates the amino acids within the cell to levels exceeding their external concentration. This pool has been called the expandable (or concentrating) pool[15] and has many of the characteristics of the amino acid-concentrating system observed in *E. coli*. The size of this pool varies with external concentration and the pool is only evident when amino acids (or analogs) are present in the medium. Material in this pool is sensitive to osmotic shock and readily exchanges with external amino acids.

A second pool, called the internal (or conversion) pool[15] is always found in exponentially-growing cells. The amino acids contained in this pool are on the main line of synthetic events. It is here that the conversion of one amino acid to others occurs, furnishing the appropriate molecules for protein incorporation. Once in this pool, these amino acids do not exchange with exogenous or accumulated amino acids, nor are they sensitive to osmotic shock. In the absence of exogenous amino acids (or analogs), this pool is formed solely from the carbon source (sugar). During exponential growth the size of this pool remains fixed and contains 13 % of the total cellular carbon. The presence of exogenous or accumulated amino acids in the cell does not alter the pool size.

In addition to these functional differences, the two pools can be extracted separately. The expandable pool alone is extractable with cold water; both pools are extractable with cold trichloroacetic acid.

Despite the existence of such amino acid pools, very little pFPhe can be *concentrated* by the yeast cells. Fig. 2 shows the total quantity of this analog contained in both pools (TCA-soluble fraction) as a function of exogenous analog concentration. These data are similar to those obtained with *E. coli* (Fig. 2), except that in the yeast cell saturation of the concentrating system occurs at a lower external level. Very little *p*FPhe is found in the metabolic pools in excess of the external concentration. In the yeast, as in *E. coli*, protein incorporation of the analog was found to be directly dependent upon pool concentration. As a consequence of this reduced capacity for accumulation, analog replacement in the yeast cell requires a higher external concentration than in *E. coli* for the same degree of protein substitution.

Competitive utilization of amino acids and amino acid analogs: In the yeast our principal interest has centered on the internal pool of amino acids, because of its necessary and essential role in the synthesis of protein. Kinetic interrelationships among the internal pool, the expandable pool and the proteins are difficult to measure, however, at low external concentrations of *p*FPhe because of the possibility of saturation of the concentrating system for the analog.

At high concentrations of DL-*p*FPhe and L-phenylalanine (10^{-3} *M* and 10^{-4} *M* respectively) added simultaneously to a growing culture of *Candida utilis*, both compounds are taken up by the cells in easily measurable quantities. Two identical flasks with reciprocal labeling ($[^{12}\text{C}]$Phe + $[^{14}\text{C}]$*p*FPhe in one and $[^{14}\text{C}]$Phe + $[^{12}\text{C}]$*p*FPhe in the other) were inoculated with cells from an exponentially-growing culture of *C. utilis*. The distribution of the analog and phenylalanine was obtained by following kinetics of incorporation of the two labeled compounds in the expandable pool, the internal pool, and the protein. The results of a typical experiment are shown in Table II.

TABLE II

DISTRIBUTION OF PHENYLALANINE AND *p*-FLUOROPHENYLALANINE IN *Candida utilis*

External ratio of DL-*p*-fluorophenylalanine to L-phenylalanine was 10:1.

	Expandable pool (mμmoles/g wet weight cells)	*Internal pool (mμmoles/g wet weight cells)*	*Protein* (mμmoles/Δ g wet weight cells)*
*p*FPhe	23.9	14.0	725
Phenylalanine	4.58	6.1	305
Ratio *p*FPhe/Phe	5.2	2.30	2.38

* Δ gram wet weight cells = mass of cells grown after the addition of the tracer materials.

The yeast cells, given an external molar ratio of analog to amino acid of 10/1, contained these materials in the expandable pool at a ratio of 5.2/1. The ratio of the analog to amino acid in the internal pool was found to be 2.3/1 *and was identical to the ratio obtained in the proteins*. It appears that no further selection occurs by the processes through which the materials of the internal pool are made into protein.

DISCUSSION

The results obtained from the studies of the kinetics of utilization of *p*FPhe indicate that in yeast at least two processes exist selecting the natural amino acid in preference to the analog for protein synthesis. It is possible to correlate these results with schemes[14–16] describing the flow of endogenously synthesized amino acid carbon, and to show the alterations in the carbon flow produced by exogenous amino acid. Fig. 6 summarizes some of the essential details.

In the absence of exogenous amino acids (or analogs), all of the amino acid requirements of the cell are derived from the sugar carbon and CO_2. "Families"[17] of amino acids are formed from "parent" members: the parent is synthesized in the amino acid production system (Fig. 6) with the conversion of the parent to related family members occurring in the internal pool[14, 15].

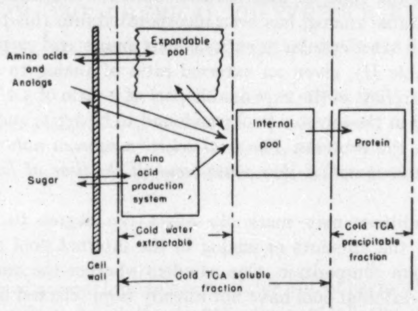

Fig. 6. Carbon flow in *C. utilis*.

The addition of amino acids or analogs to the medium results in their rapid cellular accumulation by a concentrating system (expandable pool) to levels exceeding (a) the external concentration, and (b) the intracellular level usually maintained through endogenous amino acid production from sugar. The accumulated amino acids mix with those amino acids endogenously formed, and from this mixture are withdrawn the molecules required for the internal pool and protein synthesis.

One of the first steps in the selection between an exogenous amino acid and an analog occurs in the concentrating system: With *E. coli*, accumulation of exogenous *p*FPhe is markedly reduced by the addition of equimolar quantities of phenylalanine (Fig. 3). It is evident that phenylalanine has a greater affinity for the concentrating system than *p*FPhe has. With *C. utilis*, this preference for phenylalanine is also observed (expandable pool, Table II), although not to the same extent as measured with *E. coli*.

Another process appears to be involved in the uptake of *p*FPhe, by-passing the concentrating system as shown in Fig. 6. Analog accumulation in the cell in excess of the external concentration does not occur beyond certain levels of exogenous *p*FPhe. However, with increasing external concentrations, larger and larger quantities of *p*FPhe are found in the cold TCA soluble fraction of the cell. After saturation of the concentrating system, the *p*FPhe taken up per unit volume of cells is proportional to, but smaller than, the external level (Fig. 2). Evidence supporting this interpretation is found in the data of HALVORSON AND COHEN[16]. These authors suggested that in *Saccharomyces cerevisiae* exogenous amino acids could be used for protein synthesis without equilibrating with the expandable pool.

Thus materials available for utilization in the internal pool are dependent upon a number of environmental and cellular factors. These are: (a) The ability to concentrate exogenous materials. This process depends upon the kind of exogenous supplements available, the relative specific affinity of each substrate for the concentrating mechanism, and the degree to which each substance can be accumulated (pool saturation). (b) An entry process where cellular uptake is directly proportional to the external concentration. (c) Endogenous amino acid production.

Incorporation into the internal pool of materials made available from the above processes, provides a second opportunity for the selection between an amino acid and its analog for protein synthesis. Further competition among these materials occurs at the time, or prior to the time, of incorporation into the internal pool. Once an amino acid, or an acceptable analog, has been incorporated into this pool no further dilution or exchange with other cellular or extracellular amino acid carbon occurs[14, 15].

The yeast cells (Table II), given an external ratio of analog to amino acid of 10/1, contained these materials in the expandable pool at a ratio of 5.2/1. The ratio of the analog to amino acid in the internal pool was found to be 2.3/1, and was identical to the ratio obtained in the proteins. *The final selection between natural amino acid and analog for protein incorporation thus must occur at the time of formation of the internal pool.*

Environmental conditions may markedly affect the degree to which analog substitution occurs, but the amounts of analog in the internal pool appear closely related to the final protein composition. One wonders whether the amino acids and analogs contained in the internal pool have not already been selected by the protein-forming templates, but have yet to be linked together in polypeptide strands.

ACKNOWLEDGEMENT

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REFERENCES

[1] A. B. PARDEE, V. G. SHORE AND L. S. PRESTIDGE, *Biochim. Biophys. Acta*, 21 (1956) 406.
[2] R. MUNIER AND G. N. COHEN, *Biochim. Biophys. Acta*, 21 (1956) 592.
[3] G. N. COHEN AND D. B. COWIE, *Comptes rend.*, 244 (1957) 680.
[4] D. B. COWIE AND G. N. COHEN, *Biochim. Biophys. Acta*, 26 (1957) 252.
[5] G. BRAVERMAN AND M. YCAS, *Arch. Biochim. Biophys.*, 68 (1957) 112.
[6] A. B. PARDEE AND L. S. PRESTIDGE, *Biochim. Biophys. Acta*, 27 (1958) 330.
[7] R. S. BAKER, J. E. JOHNSON AND S. W. FOX, *Biochim. Biophys. Acta*, 28 (1958) 318.
[8] G. N. COHEN AND R. MUNIER, *Biochim. Biophys. Acta*, 31 (1959) 447.
[9] R. MUNIER AND G. N. COHEN, *Biochim. Biophys. Acta*, 31 (1959) 378.
[10] D. B. COWIE, G. N. COHEN, E. T. BOLTON AND H. DE ROBICHON-SZULMAJSTER, *Biochim. Biophys. Acta*, 34 (1959) 39.
[11] A. YOSHIDA AND M. YAMASAKI, *Biochim. Biophys. Acta*, 34 (1959) 159.
[12] R. J. BRITTEN, R. B. ROBERTS AND E. F. FRENCH, *Proc. Natl. Acad. Sci., U.S.*, 41 (1955) 863.
[13] *Annual Report, Dept. Terrestrial Magnetism, Carnegie Inst. Wash.*, Washington, D.C., 1957.
[14] D. B. COWIE AND B. WALTON, *Biochim. Biophys. Acta*, 21 (1956) 211.
[15] D. B. COWIE AND F. T. MCCLURE, *Biochim. Biophys. Acta*, 31 (1959) 236.
[16] H. O. HALVORSON AND G. N. COHEN, *Ann. inst. Pasteur*, 95 (1958) 73.
[17] R. B. ROBERTS, P. ABELSON, D. B. COWIE, E. T. BOLTON AND R. J. BRITTEN, *Studies of Biosynthesis in* E. coli, Carnegie Inst. Wash. Publ. No. 607, Washington, D.C., 1955.

ABNORMAL HUMAN HAEMOGLOBINS

IV. THE CHEMICAL DIFFERENCE BETWEEN NORMAL HUMAN HAEMOGLOBIN AND HAEMOGLOBIN C

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SUMMARY

Tryptic digests of normal human haemoglobin and of haemoglobin C contain a peptide fragment (peptide 4) which apparently alone of all the peptides has a different chemical structure in the two haemoglobins. These peptides have been degraded and their amino acid sequences have been determined in the two cases. The sole alteration is the replacement of a glutamic acid residue of normal haemoglobin by lysine in haemoglobin C.

INTRODUCTION

Human haemoglobin has been shown to consist of two identical halves[1]. The globin of the human haemoglobin molecule comprises two pairs of identical polypeptide chains[2-3]. The abnormal human haemoglobin, sickle cell haemoglobin (S), was found by INGRAM[4-6] to differ from normal human haemoglobin (A) by one amino acid in each of one pair of identical polypeptide chains, that is, by one amino acid in the half molecule. We have investigated the abnormal human haemoglobin C in a similar manner, and find that it also differs from haemoglobin A by one amino acid in the half molecule. The same glutamic acid residue of haemoglobin A which is replaced by valine in haemoglobin S, has been replaced by lysine[7] in haemoglobin C. The genetic implications of this change have been dealt with previously[7].

Haemoglobin C was discovered by ITANO AND NEEL[8] together with haemoglobin S in an individual suffering from a mild form of sickle cell anaemia. The techniques employed included electrophoresis on paper and in solution where haemoglobins S and C are easily distinguishable from each other and from haemoglobin A.

Individuals have been found who are homozygous for the haemoglobin C gene and who have virtually only haemoglobin C in their erythrocytes. Family studies on other patients have indicated that they are heterozygous for the haemoglobin C

Abbreviations: DNP, dinitrophenyl; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin.

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gene and the genes for either haemoglobins A, S or the thalassaemia syndrome[9]. The haemoglobin C gene is found mostly among people of negroid descent[9], but one case of the homozygous haemoglobin C disease has been reported in a Sicilian[10]. As in the case of haemoglobin S, the mutation producing haemoglobin C is inherited as a single gene[9]. From the examination of families having the genes for both haemoglobin S and haemoglobin C, RANNEY[11] has concluded that the genes for haemoglobins S and C are allelic or closely linked; that is to say they occupy the same or closely linked loci in the chromosome (see also refs. 12, 13).

Haemoglobin C has fewer net negative charges[14] than both haemoglobins A and S. This can be demonstrated both by moving boundary and paper electrophoresis under acid (pH 6.5) and alkaline (pH 8.6) conditions. ITANO[14] calculated that at pH 8.6 the haemoglobin C molecule (mol. wt.: 66,700) has about two net negative charges fewer than haemoglobin S, and that the haemoglobin S molecule has about two net negative charges fewer than haemoglobin A. On the other hand, SCHEINBERG[15] examined the relative mobilities of haemoglobins A, S and C by paper electrophoresis over a wide pH range. He concluded that haemoglobin C has between five and eight carboxyl groups fewer per molecule than haemoglobin A. We have found[7] that there is a charge difference between haemoglobins A and C caused by the substitution of two amino groups for two carboxyl groups in the molecule. These are the only charge differences in the molecules so far detected.

ITANO[16] reported that reduced haemoglobin C has a higher solubility than reduced haemoglobin A in 2.4 M phosphate solutions, but HUISMAN *et al.*[17] find that carbonmonoxy-haemoglobin C is less soluble than carbonmonoxy-haemoglobin A and that the reduced haemoglobins A and C have about the same solubility in salt solutions. These findings might suggest that there is more than one type of haemoglobin C (just as there is more than one type[18] of haemoglobin D); however, we have examined three samples of haemoglobin C from unrelated negro patients and all have the same structure as far as can be determined by our methods.

Amino acid analyses of haemoglobins A and C have shown only small and statistically insignificant differences in their compositions[14], except that HUISMAN reported an increase in the lysine content of haemoglobin C, as would be expected from our findings. On the other hand, he did not observe a corresponding fall in the number of glutamic acid residues. Other differences between the two haemoglobins, reported by HUISMAN in the same paper, have not so far been substantiated by us.

The present paper deals with the comparison of the tryptic digests of haemoglobins A and C, the chymotryptic digests of their trypsin resistant "cores", and the structural analyses of the peptides in the haemoglobin A and C trypsin digests which were found to differ.

MATERIALS AND METHODS

Haemoglobin

The method of preparation of the haemoglobin solutions was the same as previously described by INGRAM[5], except that the haemolysates were centrifuged after dialysis. Three different samples of haemoglobin C, from persons known to be homozygous for the haemoglobin C gene, and two samples of haemoglobin A were used. One of the samples of haemoglobin C was a solution of the pure haemoglobin in aqueous glycerine and was dialysed and centrifuged as usual. Paper electrophoresis in 0.06 *M* veronal buffer at pH 8.6 showed haemoglobin C migrating as a single band with a lower mobility than both haemoglobins A and S. No other components were visible in this test.

Digestion

Tryptic digestion of the heat denatured haemoglobin and chymotryptic digestion of the trypsin resistant core were performed in a "pH stat" as previously described[5,6].

Eleotrophoresis

Pyridine acetate buffers at pH 6.4 and 3.6, using AnalaR grade pyridine–glacial acetic acid–water (25 : 1 : 225) and (1 : 10 : 90), respectively, were used for one dimensional paper electrophoretic separation of the peptides as described by INGRAM[5]. "*Fingerprints*" of the resulting peptide mixtures of haemoglobins A and C were performed "in parallel" as previously described[5].

For better resolution of peptide mixtures, the tryptic and chymotryptic digests were subjected to electrophoresis on Whatman No. 3MM paper at pH 6.4 at 20 V/cm for 1.5 h. The peptide bands were located by a 0.025 % ninhydrin in water-saturated *n*-butanol spray followed by heating at 100° for about 5 min; they were then cut out and eluted with 20 % acetic acid. The bands from haemoglobin A and C digests so obtained were next run in parallel by descending chromatography[19] with *n*-butanol–acetic acid–water (4 : 1 : 5) on Whatman No. 1 paper or by paper electrophoresis at pH 3.6.

End group analysis of peptides

1-fluoro-2,4-dinitrobenzene as 5 % solution in alcohol was added to the peptide dissolved in 1 % aqueous trimethylamine, as described by SANGER AND THOMPSON[20] for the dinitrophenylation of peptides. The DNP amino acids were determined by a modification of LEVY's two dimensional paper chromatographic system[21]. The first solvent was replaced by the *tert.*-amyl alcohol solvent saturated with the pH 6 phthalate buffer of BLACKBURN AND LOWTHER[22].

EDMAN stepwise degradation was performed by coupling the peptide with phenylisothiocyanate in the triethylamine buffer described by SJÖQUIST[23]. The phenylisothiocyanate was a commercial grade, redistilled *in vacuo*; the triethylamine was redistilled from a commercial grade, but the acetone used was of AnalaR grade and was not further purified. The PTC peptide was cyclised to the PTH of the N-terminal amino acid either in acetic acid saturated with HCl, as described SJÖQUIST[23] or in 3 *N* HCl (ref. 24). The PTH amino acid was extracted with ethyl acetate from acid or neutralized solutions, as indicated (Table IIb). When acetic acid had been used for cyclisation, the reaction mixture was first diluted with water before extraction. In order to identify the PTH amino acid, the amino acid was regenerated by heating[25,26] the PTH amino acid in concentrated HI (M.A.R.) for 4 h at 140°. The amino acid was then identified by using the two dimensional chromatographic system[27] of REDFIELD. The first (methanolic) solvent had been replaced by *n*-butanol–acetic acid–water (3 : 1 : 1). This system will be referred to as the "butanol–acetic acid: REDFIELD 2" system.

Qualitative amino acid analyses of peptides eluted from paper chromatograms were performed by hydrolysis of the peptides in hard glass capillary tubes for 14 h at 120° in constant boiling HCl which had been twice glass distilled from stannous chloride. This was followed by two dimensional chromatography in the REDFIELD system[27].

Quantitative amino acid analyses were performed in the following way. The peptides were hydrolysed as described above, and the hydrolysates chromatographed on a multisheet frame on 20 × 20 cm squares of Whatman No. 1 paper using the "butanol–acetic acid: REDFIELD 2" system. The papers were allowed to dry overnight in a fume cupboard. After development in the second dimension, the papers were dried in a Kodak film drying cabinet for 30–60 min and then steamed for 10 min to remove the excess diethylamine. The ninhydrin colour of the amino acids was developed by dipping the papers in a 5 % solution of ninhydrin in acetone which contained 5 % by volume of a 0.05 M phosphate solution (pH 7.2) (ref. 28) and by heating the papers at 65° for 22 min[29]. The spots were cut out with a razor blade and the ninhydrin colour eluted in 4-ml aliquots of 71 % ethanol. The O.D. of each spot so eluted was found by using a 1-cm cuvette in a Unicam SP 500 spectrophotometer at 575 mμ against a blank cut from one of the papers[29]. 10-μl aliquots of standard mixtures of amino acids of 2, 5, 8 and 10 mM strength were run with each determination. They contained lysine, histidine, glutamic acid, threonine, proline, valine and leucine. The amount of each amino acid in a peptide was found from the standard curves.

Partial acid hydrolysis was performed by incubation of the peptide in concentrated HCl (M.A.R.) at 37°.

Peptides T-4a and T-4b were purified by paper electrophoresis of the haemoglobin C tryptic digest at pH 6.4; peptide T-4a by electrophoresis of band 2 at pH 3.6, and peptide T-4b by paper electrophoresis of band 4 at pH 3.6 (see Fig. 4).

Tests for tryptophan and cysteine were performed by using the EHRLICH[30] and the platinic iodide[31] reagents respectively.

RESULTS AND DISCUSSION

Comparison of the haemoglobin A and C tryptic digests

The fingerprints of haemoglobin A and C digests (Fig. 1) show that in the neutral band of peptides the peptide T-4 of haemoglobin A, has been replaced in haemoglobin C by the neutral peptide T-4a. The more rigorous method of paper electrophoresis at pH 6.4 followed by chromatography (Fig. 2a and 2b) showed indeed that this was the only change in the neutral band (band 2), but in addition a new peptide (T-4b) was found in the positively charged band 4. Elution of this peptide from the chromatogram showed that it was mixed with other peptides and it was finally purified as described above.

The remaining peptides of the tryptic digests of haemoglobins A and C have been examined in the following ways: (a) By fingerprinting the digests in parallel locating the peptide spots by the 0.025 % ninhydrin spray, eluting each spot from each of two fingerprints and analysing qualitatively the amino acid composition of each and every peptide. (b) By parallel electrophoresis at pH 6.4 of the digests and parallel descending chromatography of the peptide bands (Fig. 2a and 2b). The peptides were located and qualitative amino acid analyses made as before.

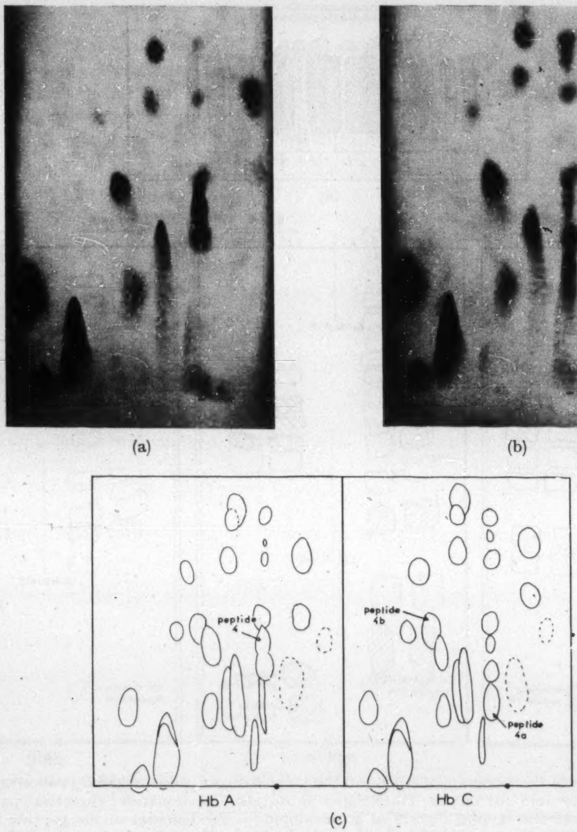


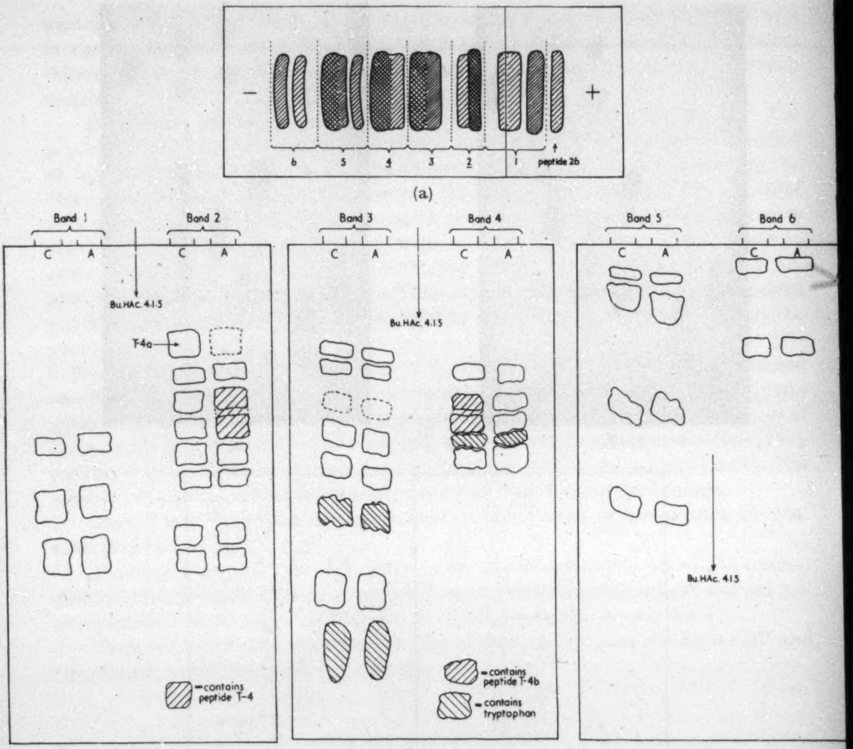
Fig. 1. Fingerprints of the trypsin digests of haemoglobins A and C. (a), Haemoglobin A; (b), Haemoglobin C; (c), Tracings showing the location of the changed peptides. The dotted lines indicate ninhydrin positive peptides which only become visible after heating.

No differences in amino acid composition, apart from the ones described in this paper, could be detected between the peptides in the tryptic digests of haemoglobins A and C.

Comparison of the chymotryptic digests of the trypsin resistant "cores" of haemoglobins A and C

Examination[6] of the "core" digests of haemoglobins A and C by fingerprinting revealed no differences in the patterns of the peptides.

Examination of the amino acid compositions of each peptide obtained by method 2 again revealed no differences between the two "cores" (Fig. 3).


−
+
6
5
4
3
2
1
peptide 2b
Band 1
Band 2
Band 3
Band 4
Band 5
Band 6
C
A
Bu.HAc. 4.1.5
T-4a
= contains peptide T-4
= contains peptide T-4b
= contains tryptophan

(a)

(bI) (bII) (bIII)

Fig. 2. (a) Tracing of the ionogram at pH 6.4 of the trypsin digest of haemoglobin A showing how the peptide bands were cut out for elution and subsequent fractionation. (Identical patterns were obtained from the trypsin digests of haemoglobin C). (b) Tracings of the peptide bands shown in Fig. 2 (a) after separation by chromatography in the *n*-butanol–acetic acid–water (4:1:5) system.

The amino acid sequence of haemoglobin A peptide T-4

The original sequence found by INGRAM[4,7] was shown to be incorrect by HUNT AND INGRAM[32], and HILL AND SCHWARTZ[33] and the corrected sequence is now:

val·his·leu·thr·pro·glu·glu·lys

Val, valine; his, histidine; leu, leucine; thr, threonine; pro, proline; glu, glutamic acid; lys, lysine. The γ-carboxyl groups of both glutamic acid residues are free and carry negative charges at neutral pH.

The amino acid sequence of haemoglobin C peptide T-4a (Fig. 1)

Equal amounts of glutamic acid and lysine were found by qualitative amino acid analysis of the peptide in the REDFIELD system as judged by eye. Tryptophan

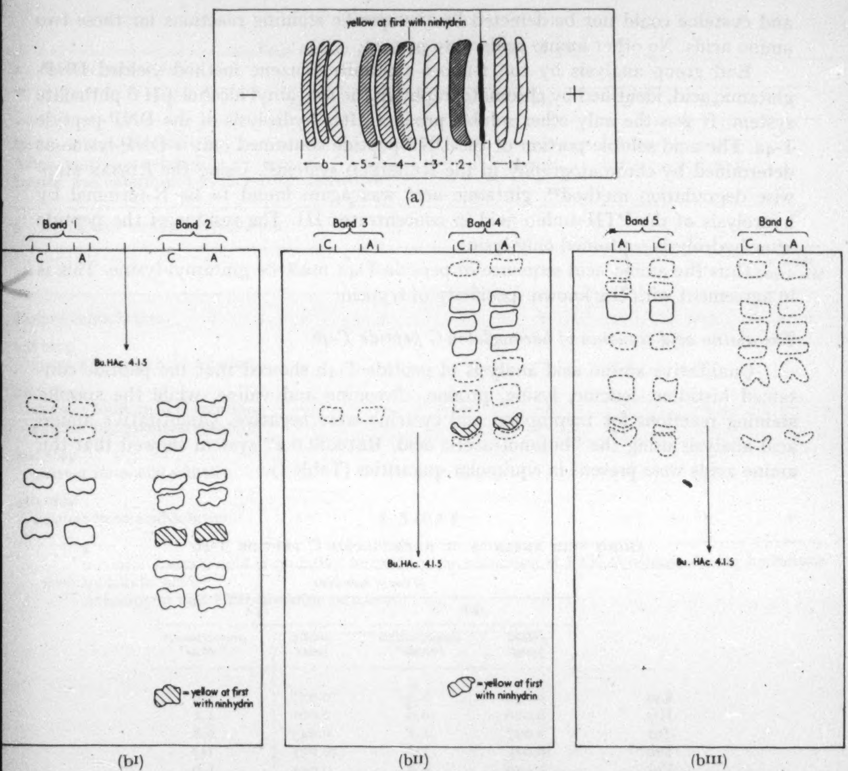

yellow at first with ninhydrin
—
+
6
5
4
3
2
1
(a)
Band 1
Band 2
C
A
C
A
Bu.HAc. 4:1:5
= yellow at first with ninhydrin
(bI)
Band 3
Band 4
C
A
C
A
Bu.HAc. 4:1:5
= yellow at first with ninhydrin
(bII)
Band 5
Band 6
A
C
C
A
Bu. HAc. 4:1:5
(bIII)

Fig. 3. (a) Tracing of the ionogram at pH 6.4 of the chymotrypsin digest of the trypsin-resistant "core" of haemoglobin (see Fig. 2(a)). (b) Tracings of the peptide bands shown in Fig. 3(a) separated by chromatography in the *n*-butanol–acetic acid–water (4:1:5) system.

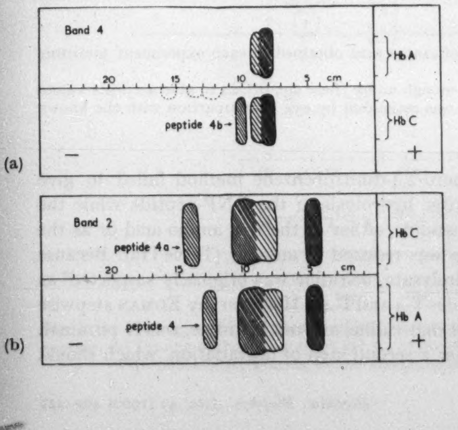

Band 4
20
15
10
5
cm
Hb A
peptide 4b →
Hb C
—
+
(a)
Band 2
peptide 4a →
20
15
10
5
cm
Hb C
peptide 4 →
Hb A
—
+
(b)

Fig. 4. (a) Tracing of the ionogram at pH 3.6 of the peptide band 2 from Fig. 2(a) of haemoglobins A and C run side by side to show the absence of the peptide A T-4 and the presence of the peptide C T-4a in haemoglobin C. (b) Tracing of the ionogramat pH 3.6 of the peptide band 4 from figure 2(1) of haemoglobins A and C run side by side to show the presence of the peptide C T-4b in haemoglobin C.

and cysteine could not be detected by the specific staining reactions for these two amino acids. No other amino acids were present.

End group analysis by the 1-fluoro-2,4-dinitrobenzene method yielded DNP-glutamic acid, identified by chromatography in the *tert.*-amyl alcohol–pH 6 phthalate system. It was the only ether soluble product after hydrolysis of the DNP-peptide T-4a. The acid soluble portion of the DNP-peptide contained only ε-DNP-lysine as determined by chromatography in the REDFIELD system[27]. Using the EDMAN stepwise degradation method[23], glutamic acid was again found to be N-terminal by hydrolysis of the PTH-amino acid in concentrated HI. The residue of the peptide after hydrolysis contained only lysine.

Thus the amino acid sequence of peptide T-4a must be glutamyl-lysine. This is in agreement with the known specificity of trypsin.

The amino acid sequence of haemoglobin C peptide T-4b

Qualitative amino acid analysis of peptide T-4b showed that the peptide contained histidine, leucine, lysine, proline, threonine and valine, while the specific staining reactions for tryptophan and cysteine were negative. Quantitative amino acid analysis using the "butanol–acetic acid, REDFIELD 2" system showed that the amino acids were present in equimolar quantities (Table I).

TABLE I

AMINO ACID ANALYSIS OF HAEMOGLOBIN C PEPTIDE T-4b

	Time of hydrolysis			
	18 h		*14 h*	
	µmoles found	*µmoles/µmoles Peptide**	*µmoles found*	*µmoles/µmoles Peptide**
Lys	0.024	1.0	0.057	1.0
His	0.020	0.9	0.061	1.1
Thr	0.027	1.2	0.045	0.8
Pro**	(0.02)	(1)	(0.05)	(1)
Val	0.020	0.9	0.054	1.0
Leu	0.023	1.1	0.065	1.2
Mean	0.023		0.056	

* Based on the mean value of µmoles of amino acid obtained in each experiment (omitting proline).

** Since the O.D. of proline is not great enough under these conditions to give accurate values the amount of proline in each hydrolysate was estimated by eye in comparison with the known standards.

End group analysis by the 1-fluoro-2,4-dinitrobenzene method failed to give any ether soluble DNP-amino acid after hydrolysis of the DNP-peptide while the residue of the peptide contained no histidine, either as the free amino acid or as the imidazole-DNP derivative, and valine was reduced in amount (Table IIa). Because of the absence of histidine in the hydrolysate, histidine was originally suspected[7] as the N-terminal amino acid in the peptides T-4 and T-4b. However, by EDMAN stepwise degradation of the peptide it was found that valine, and not histidine, was N-terminal. No amino acid could be identified after a second step of degradation, which should

TABLE II

END GROUP ANALYSIS OF PEPTIDE T-4b

a By dinitrophenylation

	Lys	*His*	*Thr*	*Pro*	*Val*	*Leu*
Before dinitrophenylation	+++	++	++	++	+++	++++
After dinitrophenylation*	++	—	+++	++	++	++++
Lysine was determined as its ε-DNP derivative*						

b By EDMAN *stepwise degradation*

	Lys	*His*	*Thr*	*Pro*	*Val*	*Leu*	*α-amino butyric acid*
Before degradation	+	+	+	+	+	+±	—
1st step							
extract from an acid solution	±	—	—	—	++	—	—
2nd step							
extract from an acid solution	—	—	—	—	±	—	—
extract from neutral solution**	—	—	—	—	—	—	—
3rd step							
extract from acid solution	±	—	—	—	—	++	±
4th step							
extract from acid solution	—	—	—	—	—	+	+

* α-Amino butyric acid is probably formed by the reduction of PTH-threonine during hydrolysis with hydriodic acid.

** Attempt to find PTH-histidine (see text).

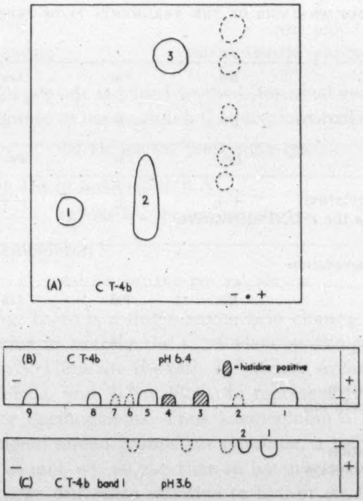


Fig. 5. (a) Tracing of the fingerprint of the partial acid hydrolysate of the haemoglobin C T-4b peptide. (b) Tracing of the ionogram at pH 6.4 of the partial acid hydrolysate of the haemoglobin C T-4b peptide. (c) Tracing of the ionogram at pH 3.6 of band 1 from Fig. 5(b).

TABLE III

PARTIAL ACID HYDROLYSIS OF PEPTIDE T-4b

a 40 h partial acid hydrolysate separated by Fingerprinting (Fig. 5a)

Peptide	*Lys*	*His*	*Thr*	*Pro*	*Val*	*Leu*
A-1	?	—	—	—	—	—
A-2	+++	—	+++	+++	—	—
A-3	—	++	—	—	++	+++

b 90 h partial acid hydrolysate separated by paper electrophoresis at pH 6.4 (Fig. 5b)

Peptide	*Lys*	*His*	*Thr*	*Pro*	*Val*	*Leu*
B-3	—	+++	—	—	+++	++++
B-4	±	++	±	±	++	—
B-5	+++	—	+++	+++	—	—
B-8	+	—	—	+	—	—
B-9	++	—	—	—	—	—

c Band B-1 separated by paper electrophoresis at pH 3.6 (Fig. 5c)

Peptide	*Lys*	*His*	*Thr*	*Pro*	*Val*	*Leu*	*Remarks*
C-1	—	—	++	±	++	+++	Probably free amino acids
C-2	—	—	++	++	—	—	

TABLE IV

END GROUP ANALYSIS OF THE FRAGMENTS FROM PEPTIDE T-4b

a By dinitrophenylation

Peptide B-3	*His*	*Val*	*Leu*	*DPN amino acid*
Residue after dinitrophenylation	—	+	++	None

Peptide B-5	*Lys*	*Thr*	*Pro*	*DPN amino acid*
Residue after dinitrophenylation	++	±	++	Threonine
Lysine was determined as the ε-DNP derivative				

b By EDMAN *stepwise degradation*

Peptide B-3	*His*	*Val*	*Leu*
Before degradation	++	++	+++
1st step			
extract from acid solution	±	++	±
extract from neutral solution	—	—	—
2nd step			
extract from acid solution	—	±	±
extract from neutral solution	—	—	—
3rd step			
extract from acid solution	—	—	+
Unhydrolysed residue	—	—	—

have given histidine, but leucine was found on the third step, as shown in Table IIb. The reason for this behaviour is not yet clear.

Partial acid hydrolysis of the peptide T-4b, derived from about 40 mg of haemoglobin, in concentrated HCl at 37° for 40 h, followed by separation of the peptide fragments obtained by fingerprinting, gave two main peptides, A-2 and A-3 (Fig. 5a). The qualitative amino acid analyses of these fragments are shown in Table IIIa. Hydrolysis of the peptide under the same conditions but for 90 h gave more fragments which were readily separated by paper electrophoresis at pH 6.4 and 3.6 (Fig. 5b and 5c). The qualitative amino acid analyses of some of these peptide fragments is shown in Table IIIb. Both peptides B-3 and B-5 were dinitrophenylated; B-3 gave no DNP amino acid and the residue contained no histidine and less valine than leucine as is shown in Table IVa. B-5 gave DNP-threonine in the ethereal extract and the residue of the hydrolysed DNP-peptide contained proline and lysine as shown in Table IVa. Three steps of the EDMAN degradation were performed on the peptide B-3 yielding valine for the first step, nothing for the second step and leucine for the third step, while the unhydrolysed residue contained no amino acids (Table IVb).

From these results the sequence of peptide T-4b can be constructed in the following way:

Composition	val$_1$ his$_1$ leu$_1$ thr$_1$ pro$_1$ lys$_1$
EDMAN degradation	val– ? –leu–(thr, pro, lys)
Partial acid hydrolysis	
A-2	(thr, pro, lys)
A-3	(val, his, leu)
B-3	val–his–leu.
B-4	(val, his)
B-5	thr–(pro, lys)
B-8	(pro, lys)
C-2	(thr, pro)
Sequence	val · his · leu · thr · pro · lys

Assuming that the T-4a peptide is joined to the C-terminal end of peptide T-4b then the sequence of the peptide in haemoglobin C equivalent to T-4 is:

val · his · leu · thr · pro · lys · glu · lys,

compared with peptide T-4 in haemoglobin A:

val · his · leu · thr · pro · glu · glu · lys

and peptide T-4 in haemoglobin S:

val · his · leu · thr · pro · val · glu · lys

It can be seen that there is a single amino acid change in haemoglobin C of a glutamic acid to a lysine in exactly the same place as the glutamic acid to valine change in haemoglobin S. These are the only differences so far detected by us in the haemoglobin half-molecule, and so are likely to represent the whole of the charge difference between the haemoglobins. Thus haemoglobin C has two less carboxyl groups and two additional amino groups per molecule, a total of four less negative charges per molecule of mol. wt. 66,700 than in haemoglobin A. SCHEINBERG[15] had estimated that the charge difference was due to five or six fewer carboxyl groups. He came to this conclusion because he was unable to detect a difference in electrophoretic mobility at low pH between molecules of haemoglobin A and C with positive charges of about 110 and 112. It is unlikely that his method would be sensitive enough.

One of the most striking differences in the properties of haemoglobins A, S and C are their solubilities in the deoxygenated form. Since the only detectable differences in the molecules so far found are the amino acid substitutions, it is quite likely that these cause the difference in the solubilities. Conceivably the charge differences affect the pattern of charges on the surface of the molecules, so as to alter the aggregation of the molecules under conditions of high salt concentrations, either facilitating or hindering precipitation. This suggested mechanism will have to serve until the detailed three dimensional structure of haemoglobin is known and understood. The fact that haemoglobin C is able to replace haemoglobin S up to 50 % without inhibiting the gelling of the reduced haemoglobin S solution, while haemoglobin A will only replace[34] up to 25 % of haemoglobin S, is still to be explained.

Both haemoglobins S and C are found close together in Africa, and it has been suggested by MOURANT[35] that the haemoglobin C mutation has arisen from the haemoglobin S mutation, although there is no evidence to support this theory. Genetically haemoglobins A, S and C are of great interest because they illustrate biochemically the effects of allelism. These results should also be viewed in conjunction with haemoglobin G (Neel). This haemoglobin has been found by HILL AND SCHWARTZ[33] to change in the same peptide T-4, but in this case it is the second glutamic acid residue which changes to a glycine.

The present experiments show not only that a gene mutation can affect just a single amino acid residue, but that individual mutations can alter the same amino acid in polypeptide chain in different ways.

ACKNOWLEDGEMENTS

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REFERENCES

[1] M. F. PERUTZ, A. M. LIQUORI AND E. EIRICH, *Nature*, 167 (1951) 929.
[2] H. S. RHINESMITH, W. A. SCHROEDER AND L. PAULING, *J. Am. Chem. Soc.*, 79 (1957) 4682.
[3] H. S. RHINESMITH, W. A. SCHROEDER AND N. MARTIN, *J. Am. Chem. Soc.*, 80 (1958) 3358.
[4] V. M. INGRAM, *Nature*, 180 (1957) 326.
[5] V. M. INGRAM, *Biochim. Biophys. Acta*, 28 (1958) 539; 36 (1959) 402.
[6] J. A. HUNT AND V. M. INGRAM, *Biochim. Biophys. Acta*, 28 (1958) 546.
[7] J. A. HUNT AND V. M. INGRAM, *Nature*, 182 (1958) 1062.
[8] H. A. ITANO AND J. V. NEEL, *Proc. Natl. Acad. Sci. U.S.*, 36 (1950) 613.
[9] W. W. ZUELZER, J. V. NEEL AND A. R. ROBINSON, *Progr. Haematol.*, 1 (1956) 91.
[10] L. DIGGS, A. P. KRAUS, D. B. MORRISON AND L. P. RUDNICKI, *Blood*, 9 (1954) 1172.
[11] H. M. RANNEY, *J. Clin. Invest.*, 33 (1954) 1364.
[12] J. V. NEEL, *New Engl. J. Med.*, 256 (1957) 161.
[13] H. LEHMANN, *Acta Genet.*, 6 (1956/7) 413.
[14] H. A. ITANO, *Advances in Protein Chem.*, 12 (1957) 215.
[15] J. H. SCHEINBERG, *Hemoglobin*, Natl. Acad. Sci., Washington, 1958.

[16] H. A. Itano, *Arch. Biochem. Biophys.*, 47 (1953) 148.
[17] T. H. J. Huisman, P. C. van der Schaaf and A. van der Saar, *Blood*, 10 (1955) 1079.
[18] S. Benzer, V. M. Ingram and H. Lehmann, *Nature*, 182 (1958) 852.
[19] S. M. Partridge, *Biochem. J.*, 42 (1948) 238.
[20] F. Sanger and E. O. P. Thompson, *Biochem. J.*, 53 (1953) 353.
[21] A. L. Levy, *Nature*, 174 (1954) 126.
[22] S. Blackburn and A. G. Lowther, *Biochem. J.*, 48 (1951) 126.
[23] J. Sjöquist, *Arkiv Kemi*, 11 (1957) 129.
[24] H. Fraenkel-Conrat and J. I. Harris, *J. Am. Chem. Soc.*, 76 (1954) 6058.
[25] G. Schramm, G. Braunitzer and J. W. Schneider, *Nature*, 76 (1955) 456.
[26] D. F. Elliot and W. S. Peart, *Biochem. J.*, 65 (1957) 246.
[27] R. R. Redfield, *Biochim. Biophys. Acta*, 10 (1953) 344.
[28] V. M. Ingram and M. J. R. Salton, *Biochim. Biophys. Acta*, 24 (1956) 9.
[29] R. E. Kay, D. C. Harris and C. Entenman, *Arch. Biochem. Biophys.*, 63 (1956) 14.
[30] I. Smith, *Nature*, 171 (1951) 43.
[31] G. Toennies and J. J. Kolb, *Anal. Chem.*, 23 (1951) 823.
[32] J. A. Hunt and V. M. Ingram, *Nature*, 184 (1959) 640.
[33] R. J. Hill and H. C. Schwartz, *Nature*, 184 (1959) 641.
[34] A. C. Allison, *Biochem. J.*, 65 (1957) 212.
[35] A. E. Mourant, *The Distribution of Human Blood Groups*, Blackwell Oxford, 1954, p. 184.

DEOXYPENTOSE NUCLEIC ACIDS

XI. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: CONDUCTIVITY AND MOBILITY MEASUREMENTS

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SUMMARY

Various electrical properties have been derived from conductivity and electrical transport experiments for aqueous solutions of DNA. The specific conductivity shows a discontinuity when plotted against DNA concentration. Below this critical concentration the material transport number falls rapidly with decreasing concentration. The critical concentration corresponds to a similar discontinuity observed in the variation of O.D. with DNA concentration, measured at 2590 Å. A possible explanation of this phenomenon involves the denaturation of DNA at concentrations below the critical concentration. Above this concentration zone native DNA predominates with a charge fraction of 0.4 while below this concentration denatured DNA exists with a very high charge, but lowered mobility.

Abbreviation: DNA, deoxyribonucleic acid.
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INTRODUCTION

Although it has now been well established that DNA in solution behaves as a relatively stiff coil and does not undergo the morphological changes with ionisation observed with synthetic polyelectrolytes, variations of the charge of the DNA ion must produce some significant changes in flexibility. It is therefore desirable to know the charge on the nucleate ion under various conditions of ionic strength and pH.

The charge on the DNA ion has previously been determined from either membrane potential measurements or from the electrophoretic mobility. Both these methods are subject to criticism, the first because of the high DNA concentrations required to obtain measurable potentials and the lack of reproducibility of the results, the second in that the calculation of charge from electrophoretic mobility measurements requires assumptions concerning the size and shape of the kinetic unit. Values of the charge fraction (net charge per phosphorus atom) obtained from membrane potential measurements[1,2] vary from 0.4 to 0.5 at high ionic strength (0.1 or 0.2) to 0.2 at ionic strength approaching zero. The electrophoretic measurements[3] indicate a value of 0.2 which appears to be almost independent of the ionic strength. These values of the charge show that the net negative charge is less than the theoretical value of one electronic charge on every phosphorus atom, a result which has been explained by the association of sodium ions into the DNA kinetic unit. Similar behaviour has been observed with solutions of synthetic polyelectrolytes[4–7] and also for bovine serum albumen[8].

In this investigation the charge on the nucleate ion has been determined from conductivity and electrical transport measurements, using the methods described by HUIZENGA, GRIEGER AND WALL[4] for a study of the polyacrylate ion. The electrical properties of DNA solutions were studied as a function of DNA concentration in the absence of added electrolyte and, as was to have been expected from the observed denaturation of DNA at low ionic strengths[9–11], the results of this investigation are complicated by the presence of a denaturation phenomenon.

EXPERIMENTAL

The DNA used in this investigation was prepared from calf thymus glands by the detergent method[12]. The preparations were the salt free samples 2, 3, 4 and 7 which have been described in an earlier investigation[13]. The atomic extinction coefficients of these samples, measured in 0.1 *M* NaCl at 2590 Å, were found to be 6650, 6650, 6710 and 6610 respectively. The absence of sodium chloride in preparation 2, which was the preparation mainly used in this investigation, was confirmed by analysis. Solutions of DNA were prepared by dissolving the solid DNA in water at 4° to give a final concentration of $3 \cdot 10^{-3}$ *M* with respect to phosphorus. The various dilutions were made by the addition of water to the stock solutions. All concentrations were determined by phosphorus analysis on each stock solution.

A conductivity bridge, without a Wagner earthing system, was used. The oscillator was isolated from the bridge by an astatically wound shielded transformer and, since the supply was not resistively connected to ground, one side of both the cell and the bridge could be earthed. Measurements were made at 1 kc/sec. The water used throughout has a specific conductivity of $1 \cdot 10^{-6}$ ohm^{-1} cm^{-1}. All measurements were made at $25.00 \pm 0.05°$.

Transport measurements and the calculation of the derived electrical properties were made using the method described by HUIZINGA, GRIEGER AND WALL[4]. Endosmotic flow was eliminated by an opposing pressure and the changes in DNA concentration in the electrode compartments were determined by phosphorus analysis. Transport measurements were made on sample 2 only. All electrical properties refer to the Grund molecular unit.

RESULTS AND DISCUSSION

The variation of the specific conductivity and material transport number (the ratio of increase of DNA phosphorus in anode compartment to the total equivalents of electricity passed) with DNA concentration are shown in Fig. 1. The specific conductivity, as a function of DNA concentration, shows a definite discontinuity over a short range of concentration at $2\text{–}3 \cdot 10^{-4}\,M$ DNA. The material transport number rises rapidly at concentrations above the discontinuity and finally levels off at a value greater than unity. Material transport numbers greater than one infer that a proportion of the sodium ions are associated so strongly with the polyion kinetic unit that they move with it in an electric field thus producing a negative sodium ion material transport number. This result is in agreement with the earlier deductions concerning gegenion association with DNA[1-3] and with partially neutralized polyacrylic acid[4]. It can be seen that the gegenion association process only becomes apparent at concentrations above the discontinuity (in future designated as the critical concentration).

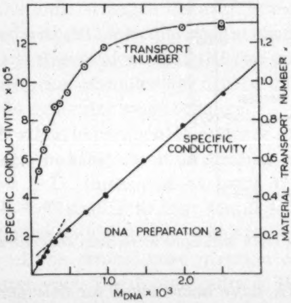

Fig. 1. The specific conductivity and material transport numbers of aqueous DNA solutions at various concentrations.

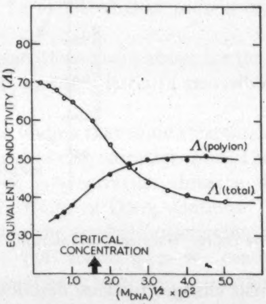

Fig. 2. The total equivalent conductivity and the nucleate ion equivalent conductivity in aqueous solution.

These results were combined, using smoothed electrical transport values and average values of the specific conductivity from preparations 2,3, 4 and 7, to derive the more fundamental electrical properties. In Fig. 2 the variation of the total and polyion equivalent conductivities with concentration are compared. Although the total equivalent conductivity decreases with increase of concentration above the critical concentration, the contribution from the polyion unit increases. This result may also be attributed to the association of sodium ions with the polyion. The contribution of the sodium ions to the conductivity of the solution is thus decreased and there is therefore a net reduction in the total equivalent conductivity. The changes that occur in the charge fraction with changing DNA concentration are shown in Fig. 3. A large increase in charge occurs on decreasing the DNA concentration below the critical value and there is a simultaneous decrease in the mobility in this concentration range. The mobility, however, in this case, is not dependent on the charge carried by the kinetic unit (mobility α polyion equivalent conductivity/charge fraction), but is a true indication of the frictional forces opposing the movement of the polyion unit in solution.

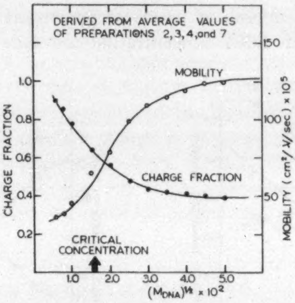

Fig. 3. The charge fraction and mobility of aqueous DNA solutions at various concentrations.

Similar changes to those described for DNA have been found for detergent solutions[14–16] which display a critical micelle concentration (CMC). The increased mobility above the CMC has, in this case, been explained in terms of aggregation such that the aggregate units (micelles), which form above the CMC, experience less frictional drag than do the same number of molecular components in the unaggregated state. Notwithstanding the improbable shape changes that native DNA would have to undergo to produce the changes in electrical properties described above, it would appear that some form of aggregation or disaggregation phenomenon is also in operation in the case of aqueous DNA solutions. Two alternative explanations appear possible.

1. The aggregation process may be similar to that found for detergent ions. Two or more DNA units would aggregate in such a way that the resulting kinetic unit offers less frictional resistance than that experienced by the constituent DNA units separately. Such a process should result in a reversible single molecule–aggregate system, because there is no reason to suspect that these molecules would be aggregated by a specific type of linkage. This process would also involve a molecular weight change at the critical concentration.

2. The aggregation phenomenon might be of entirely different origin if the intact native DNA unit is classed as the aggregate. Passage through the critical concentration on decreasing the DNA concentration could then represent the disaggregation of the native DNA double helix by hydrogen bond cleavage between the paired nitrogen

bases. This would eliminate the force holding the two spirals in register, and the molecule would then be in what is known as the denatured state. As the hydrogen bonds between the base pairs are thought to be highly specific, such a disaggregation process described above should be quite distinct from the former process (1) in being irreversible. Some ambiguity arises when the molecular weight changes are considered. Once the specific hydrogen bonds have been broken, there is no direct linkage between the two spirals, but, due to the plectonemic nature of the interwound strands, no decrease in molecular weight can immediately take place. In the case of denaturation by acid[17], alkali[18], thermal treatment[19] and low ionic strength[11], no change in molecular weight has been found (when measured at high ionic strength), as compared with the native sample. On the other hand, once all the hydrogen bonds have been broken, unwinding of the two chains is apparently energetically feasible[20]. It is possible that one of the more important factors which determine whether the spirals do unwind is the presence of a few remaining native hydrogen bonds or of some reformed hydrogen bonds of a non-specific nature. However, by analogy with acid, alkali, thermal, and low ionic strength denaturation, it would be expected that process (2) would proceed without molecular weight change.

It is thus evident that the two possible explanations given above for the critical concentration phenomenon can be differentiated by the criteria of reversibility and possibly molecular weight change.

Further, experimental evidence supports the finding that some structural change occurs in the kinetic unit on diluting below the critical concentration zone. The variation of O.D. (measured at 2590 Å) with DNA concentration shows a discontinuity[11, 21, 22] similar to that found for the conductivity of DNA solutions. This discontinuous optical property has been confirmed in the present investigation and the value of the critical concentration agrees with that found from the conductivity measurements. The discontinuity in the u.v. absorption is such that below the critical concentration the extinction coefficient increases.

In view of the fact that denaturation has already been shown to occur on reducing the ionic strength[9, 10] and by dissolution in water at low DNA concentrations[11], it would appear that the mechanism (2), involving hydrogen bond cleavage, is the more likely explanation of the critical concentration phenomenon. The increase in extinction coefficient on dilution below the critical concentration would also be compatible with a denaturation process.

If the critical concentration phenomenon does arise from a denaturation process. then the electrical properties described above will refer to native DNA at high concentrations only. Under such conditions the value of the charge fraction is 0.40 (Fig. 3) for the native material in the absence of added electrolyte. This is to be compared with the values previously reported of 0.2 and 0.4 at zero or low ionic strength[1–3]. The denatured material (at concentrations below the critical zone) apparently has a much higher charge, which approaches the theoretical maximum value. The mobility of the denatured material has decreased as compared with the native DNA. Although the charge increases on passing from the native to the denatured state, the equivalent polyion conductivity decreases; this arises from the large mobility decrease which accompanies this process.

Although it would be expected that denaturation would be accompanied by a general molecular collapse, as indeed has been shown for denatured DNA in electrolyte solutions[11] at high ionic strength, this does not appear to be the case for denaturation caused by dilution in salt free solutions. It has been found that denaturation in aqueous solution leads to a highly charged unit and it is thought that, in this instance, an intramolecular repulsion acts counter to the collapsing tendency brought about by the rupture of the hydrogen bonds. The large size of the kinetic unit could possibly explain the low mobility observed for denatured DNA.

Further investigations will be necessary to determine whether denaturation by dilution of aqueous DNA solutions can completely explain all the electrical properties reported above.

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REFERENCES

[1] J. M. Creeth and D. O. Jordan, *J. Chem. Soc.*, (1949) 1409.
[2] J. Shack, R. J. Jenkins and J. M. Thompsett, *J. Biol. Chem.*, 198 (1952) 85.
[3] A. Mathieson and J. V. McLaren, *J. Chem. Soc.*, (1956) 303.
[4] J. R. Huizenga, P. F. Grieger and F. T. Wall, *J. Am. Chem. Soc.*, 72 (1950) 2636.
[5] F. T. Wall and R. H. Doremus, *J. Am. Chem. Soc.*, 76 (1954) 868.
[6] F. T. Wall and R. H. Doremus, *J. Am. Chem. Soc.*, 76 (1954) 1557.
[7] F. T. Wall and M. Eitel, *J. Am. Chem. Soc.*, 79 (1957) 1550.
[8] R. H. Doremus and P. Johnson, *J. Phys. Chem.*, 62 (1958) 203.
[9] R. Thomas, *Biochim. Biophys. Acta*, 14 (1954) 231.
[10] P. D. Lawley, *Biochim. Biophys. Acta*, 21 (1956) 481.
[11] L. F. Cavalieri, M. Rosoff and B. H. Rosenberg, *J. Am. Chem. Soc.*, 78 (1956) 5239.
[12] E. R. M. Kay, N. S. Simmons and A. L. Dounce, *J. Am. Chem. Soc.*, 74 (1952) 1724.
[13] J. H. Coates and D. O. Jordan, *Biochim. Biophys. Acta*, 35 (1959) 309.
[14] M. E. L. McBain, W. B. Dye and S. M. Johnston, *J. Am. Chem. Soc.*, 61 (1939) 3210.
[15] K. J. Mysels and C. I. Dulin, *J. Colloid Sci.*, 10 (1955) 461.
[16] G. S. Hartley, B. Collie and C. S. Samis, *Trans. Faraday Soc.*, 32 (1936) 796.
[17] E. P. Geiduschek, *J. Polymer Sci.*, 31 (1958) 67.
[18] P. Ehrlich and P. Doty, *J. Am. Chem. Soc.*, 80 (1958) 4251.
[19] S. A. Rice and P. Doty, *J. Am. Chem. Soc.*, 79 (1957) 3937.
[20] C. Levinthal and H. R. Crane, *Proc. Natl. Acad. Sci., U.S.*, 42 (1956) 436.
[21] J. Pouyet, G. Schiebling and H. Schwander, *J. chim. phys.*, 47 (1950) 716.
[22] M. Dhaussy, *J. chim. phys.*, 52 (1955) 150.

DEOXYPENTOSE NUCLEIC ACIDS

XII. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: CHANGES PRODUCED BY ENVIRONMENT

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SUMMARY

An investigation of salt free DNA solutions has yielded further information on the critical concentration phenomenon reported earlier. This phenomenon appears to arise from denaturation by dilution below a certain critical concentration. The critical concentration can be arbitrarily set at $21 \pm 2 \cdot 10^{-5}\,M$ DNA for several salt free DNA preparations. Conductivity measurements show, however, that the structural transition actually occurs over a concentration zone extending from 50 to $4 \cdot 10^{-5}\,M$ DNA. The process is at least in part irreversible.

The effects, on the phenomenon, of ionic strength, gegenion radius, dielectric constant, EDTA and thermal denaturation were investigated. The results reported on the effect of thermal denaturation in particular support the premise that the critical concentration originates from a denaturation process. These findings are in agreement with the changes observed by CAVALIERI *et al.*[4], who find a large decrease in mean end-to-end distance on dilution of salt free DNA solutions to low concentrations. This change was not accompanied by a light scattering molecular weight change.

INTRODUCTION

In a previous paper changes of the charge, mobility and transport number of the DNA kinetic unit were reported as a function of the polyion concentration in salt free solution[1]. The rapid changes of these properties over a small concentration zone could be explained by two alternative mechanisms; one involving aggregation of two or more DNA polyions and the other involving the dissociation of the DNA double helix yielding, most probably, two entangled polynucleotide chains. The latter process is usually referred to as denaturation. Denaturation must be the more likely explanation in view of the reported denaturation at low ionic strength[2,3] and in dilute aqueous solution[4]. Experiments are described in this investigation which were designed to test the effect of various environmental factors on the critical concentration phenomenon.

Abbreviations used: DNA, deoxyribonucleic acid; EDTA, ethylene diamine tetra-acetic acid.

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EXPERIMENTAL

The samples of calf thymus DNA used in this investigation have already been described[5]. All DNA concentrations were determined by phosphorus analysis on stock solutions ($3 \cdot 10^{-3}$ M DNA in water). The stock solutions were first centrifuged to remove dust particles.

Conductivity measurements were made with the bridge already described[1]. In the study of the conductivity at various DNA concentrations, increases in concentration were effected in the conductivity cell in the following way. Successive additions of a concentrated stock solution ($3 \cdot 10^{-3}$ M DNA) were made to a known volume of water or DNA solution by first withdrawing an equal volume of liquid from the cell. The contents were then mixed by a stream of purified nitrogen (free from carbon dioxide). Dilution of a concentrated solution was made in a similar way, by removal of solution and the addition of an equal volume of water. The former method of preparing solutions (*i.e.*, by increasing the concentration) was in general preferred, since this procedure enabled the large water corrections, which were necessarily incurred in the investigation, to be known more precisely at the lower concentrations. This is so because such solutions will still contain a large proportion of the water of known conductivity which was present in the cell at the beginning of the experiment.

The method used to calculate the resulting DNA concentration after withdrawal and addition of known volumes of DNA solution assumed that no volume change occurred on mixing. No large errors came about through this assumption. In the two experiments where a check was made on the final concentration after, in one case 18 and in the other 20, successive increases in concentration, the final calculated concentrations were in error by 1.5 % and 2.0 % respectively as, judged by phosphorus analysis on the final solutions. An error of this magnitude does not alter the conclusions reached in this investigation.

The determination of the critical concentration was found by measurements of the specific conductivity at various increasing DNA concentrations, 15 to 20 successive increases in concentration were needed to define the critical concentration for the comparisons made in this and later investigations.

All the conductivities reported for DNA were obtained by subtraction of the conductivity contributions of the water and of any additive. The conductivity of the water was always less than $0.5 \cdot 10^{-6}$ ohms^{-1} cm^{-1} and had an average value of $0.35 \cdot 10^{-6}$ ohms^{-1} cm^{-1}.

Sedimentation experiments were made on a Spinco Model E Ultracentrifuge equipped with an u.v. absorption optical system. The photographic records were converted into plots of photographic density *versus* distance from the centre of the rotor using an Analytrol photodensitometer.

RESULTS AND DISCUSSION

Detailed investigation of the conductivity at various DNA concentrations

The variation of specific conductivity with DNA concentration has been found to exhibit a discontinuity at a critical concentration of $2\text{–}3 \cdot 10^{-4}$ M DNA[1]. Fig. 1 shows the results of a detailed study of this phenomenon. The difference between the conductivities obtained by increasing and decreasing the DNA concentration will be discussed later. The inset plot of Fig. 1 represents the lower concentration range. It can be seen that the critical concentration is more accurately represented by a zone of concentration; above and below this zone a linear relation appears to exist. According to an earlier investigation[1] the linear relation above the critical zone is preserved up to at least $2.5 \cdot 10^{-3}$ *M* DNA. The average critical concentration (the point at which the two linear portions intersect) for the salt free preparations 2, 3, 4 and 7 (see ref. 5) was found to be $21 \pm 2 \cdot 10^{-5}$ *M* while the actual zone extended from 4–$50 \cdot 10^{-5}$ *M*. All these preparations gave values of 80 ± 10 for the equivalent conductivity at zero concentration. Another preparation, number 1, gave higher conductivities and a lower critical concentration. The higher conductivity is almost certainly due to the salt impurity in this preparation[5] and the lower critical concentration is also to be expected in view of the dependence of the critical zone on ionic strength, to be discussed later.

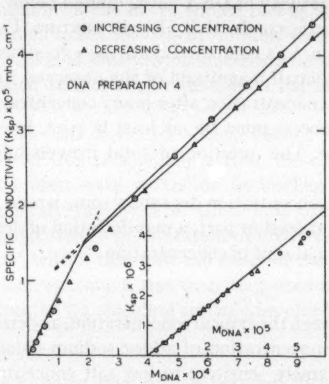

Fig. 1. Specific conductivity of salt free DNA solutions at various concentrations. DNA preparation 4. (a) Obtained by progressive increase in concentration (addition of concentrated stock solution). (b) Obtained by progressive dilution of DNA solution with water. Insert graph shows the linear relation that exists below the critical concentration zone.

To show that any possible frequency dependent errors were not responsible for the critical concentration phenomenon, conductivities were measured at 1, 2.5, 10, and 20 kc/sec and the values obtained extrapolated to infinite frequency and compared with the results obtained at 1 kc/sec. From this comparison it was evident that change in frequency had little effect on the critical concentration phenomenon.

A test for the reversibility of the phenomenon was carried out by making conductivity measurements on DNA solutions prepared by both increasing and decreasing the DNA concentration (Fig. 1). The small but significant increased conductivity of the plot obtained when increasing the DNA concentration (curve (a), Fig. 1) over that obtained on decreasing the concentration (curve (b), Fig. 1) is consistent with irreversibility. A small amount of reversibility cannot, however, be excluded, because the method used to increase the DNA concentration (addition of concentrated DNA solution) tends to mask any irreversible changes that occur at the critical concentration. Calculations show that increasing the concentration from below the critical concentration zone to $1 \cdot 10^{-3}$ *M* (by addition of concentrated DNA solution) results in a solution containing only 3.8 % of the original DNA present at concentrations below the critical zone. Using an equivalent conductivity of 80 for the DNA present that had been below the critical concentration and 43.5 for the remainder (obtained from the conductivity of DNA never diluted below the critical zone, Fig. 1, curve (b)), results in a specific conductivity for the mixture (Fig. 1, curve (a)) at $1 \cdot 10^{-3}$ *M* of $0.45 \cdot 10^{-5}$ ohm^{-1} cm^{-1} as compared with the experimental value of $0.46 \cdot 10^{-5}$ ohm^{-1} cm^{-1}. Due to the small magnitude of the observed irreversibility and due to the uncertainty in DNA concentration after many concentration changes it may only be concluded that the process must be, at least in part, irreversible, but not necessarily totally irreversible. The question of total irreversibility must await a more sensitive test.

Because the critical concentration does show some irreversibility, it would appear that the phenomenon is, at least in part, a manifestation of denaturation which occurs on dilution through a critical zone of concentration.

Influence of ionic strength

The relationship between the critical concentration, as determined by conductivity measurements, and the concentration of added sodium chloride is shown in Fig. 2. The phenomenon is extremely sensitive to low salt concentrations, the addition of $2 \cdot 10^{-5}$ *M* NaCl being sufficient to lower the critical concentration by one-third. The fact that salt lowers the critical concentration is compatible with both processes that have been proposed to explain the phenomenon. If the critical zone resulted from denaturation, then the addition of salt should lower the critical zone, because the presence of shielding ions is known to protect the polyion in this respect[2–4], by decreasing the electrostatic repulsion between the charged sites on the molecule. The alternative, less likely, explanation of the phenomenon involving aggregation of two or more DNA molecules at concentrations higher than the critical zone also receives support from the findings concerning the effect of salt. A shielding action would tend to lower the repulsive forces that must be present between the charged polyions and would therefore increase the possibility of aggregation. The results given in Fig. 2 indicate that marked changes in this critical concentration can be produced by the addition of small amounts of salt; it is therefore evident that investigations of aqueous DNA solutions are liable to be irreproducible unless salt impurity is rigorously excluded.

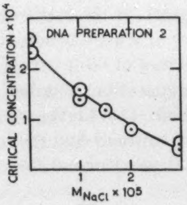

Fig. 2.

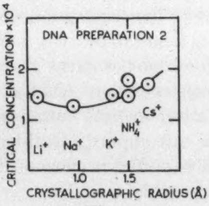

Fig. 3.

Fig. 2. Dependence of critical concentration on added sodium chloride. DNA preparation 2.

Fig. 3. Dependence of critical concentration on crystallographic radii of added monovalent gegenions. DNA preparation 2.

Further investigations were made on the effect of variations in size of the cation and the results are given in Fig. 3 for monovalent ions. Although the effect of cation radius is not large, it appears that the sodium ion lowers the critical concentration to the greatest extent. If it is assumed that the action of the added salt is one of shielding, then the minimum in the curve shown in Fig. 3 is not unexpected. It is known that a critical radius exists for ions in solution such that ions smaller than a certain size acquire an hydrated sheath which increases the aqueous radius of the ion[6]. If the lithium ion has such an increased radius, then the minimum in the curve can be understood.

To determine whether the decreased critical concentration, which follows the addition of salt, originated from a shielding action, the effects of much larger ions were studied. The salts used were rosaniline hydrochloride and 5-aminoacridine hydrochloride. Unfortunately the effect of these ions can only be studied over a limited concentration range due to precipitation of a DNA complex at relatively low ionic strengths. However, within this limit the effect of the large cations, in lowering the critical concentration, was much less than that shown by the small gegenions. Such a result is to be expected if a shielding action is involved.

Influence of dielectric constant

In view of the well known relationship between dielectric constant and association in simple electrolytes, experiments were made to determine the effect of changes in association between gegenion and polyion on the critical concentration phenomenon. The changes in dielectric constant were effected by various methanol–water and ethanol–water solvents. It was found that the critical concentration was lowered on decreasing the dielectric constant. Again this result is consistent with a gegenion shielding action. That association of the gegenions by the polyion increases on lowering the dielectric constant was inferred from the observed lowering of the equivalent conductivity of DNA with decreasing dielectric constant. Complete association could

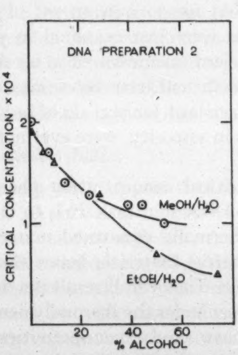

Fig. 4. Dependence of critical concentration on dielectric constant. Dielectric constant varied by the use of MeOH–H_2O and EtOH–H_2O mixtures. DNA preparation 2.

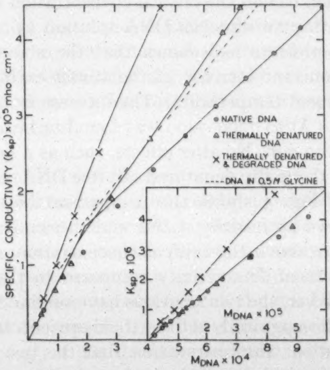

Fig. 5. Comparison of the critical concentration phenomena displayed by an initially native sample, thermally denatured DNA and DNA in 0.5 *M* glycine. Insert graph shows the lower concentration region. DNA preparation 2.

be expected in pure ethanol solvent as judged by the decreasing equivalent conductivity of DNA in increasing ethanol–water solvent ratios. This was confirmed by direct measurement of the conductivity of DNA in 98 % ethanol prepared by dialysis of an aqueous solution against increasingly stronger alcohol solutions[7]. The conductivity of DNA in such a solution was about 0.01 times that of an aqueous DNA solution. No sudden change was found in the variation of the conductivity with alcohol concentration as would be expected from the observed rapid change in sedimentation constant at 65 % ethanol[7].

The effect of a small increase in dielectric constant was investigated by conductivity measurements on DNA in the presence of 0.5 M glycine. The expected small increase in critical concentration was observed. At concentrations below the critical zone both aqueous DNA solutions and solutions in 0.5 M glycine appeared to have similar conductivities, however, at higher DNA concentrations the specific conductivity of DNA in 0.5 M glycine was found to be much greater (see Fig. 5). The possible reason for this behaviour will be discussed later.

The effect of added EDTA

As aggregation of the nucleate ion could possibly occur through divalent ion bridges, the effect of the addition of EDTA was studied. The presence of EDTA should remove from solution all divalent ions which might be present as trace impurities. EDTA at $1 \cdot 10^{-5}$ M (disodium salt) had no effect on either the critical concentration or the specific conductivity of DNA. Divalent ion bridges therefore would not appear responsible for the critical concentration phenomenon.

The effect of thermal denaturation

It is now well established that denaturation of DNA can result from thermal agitation[8,9]. As the most likely explanation of the critical concentration phenomenon rests on a similar process, it is of interest to determine what effect previous denaturation has on the critical concentration zone. Thermal denaturation was achieved by heating an aqueous DNA solution ($3 \cdot 10^{-3}$ M DNA) for 30 min at 75°. Previous experiments had shown that the above conditions were just maximal in yielding a constant increase of the atomic extinction coefficient (as measured in 0.1 M NaCl at room temperature). The increase in extinction coefficient from 6660 (measured at 2590 Å) to 7300–7400 was found to be essentially constant for periods of heating up to 200 min. No after effects, such as a slow increase in viscosity, were ever noticed in the thermally denatured salt free DNA solutions.

Fig. 5 shows the comparison between the critical concentration phenomena shown by initially native and thermally denatured DNA solutions. It is to be noted that, above the critical concentration zone, the thermally denatured material has increased conductivity compared to the initially native material; below this zone, however, the two solutions have similar conductivities. The overall result is a decrease in the magnitude of the critical concentration phenomenon for the thermally denatured solution. The observation that the two solutions have similar conductivities below the critical zone only is strong evidence that this zone itself also originates from a denaturation process. The small discontinuity that still exists in the variation of conductivity with concentration for the thermally denatured material (Fig. 5) may mean that thermal denaturation, under the conditions described earlier, was not complete and that further denaturation takes place on dilution below the critical zone. However, this appears to be an unlikely explanation in view of the constancy of the extinction coefficient and also from the similarity of this property with that obtained from thermal denaturation in salt solutions. A more likely explanation for this remaining discontinuity may arise from the possible reformation of non-specific hydrogen bonds above the critical concentration. In Fig. 5 there is also shown, for comparison, the results of measurements made on a DNA solution subjected to drastic thermal treatment (90°, for 12 h). The conductivity of this solution does not show a discontinuity but rather, the conductivity has increased over the entire concentration range. It is evident that some degradation in addition to denaturation has taken place in this solution.

The large increase in conductivity of DNA in 0.5 *M* glycine (discussed earlier and shown in Fig. 5) also occurred only above the critical concentration. Furthermore, the variation of conductivity with concentration for the thermally denatured material and for DNA in 0.5 *M* glycine are similar. It may thus be inferred that denaturation has taken place in glycine solutions at concentrations above the critical zone, and 0.5 *M* glycine is to be considered a denaturing agent for DNA at zero ionic strength.

It has been reported that a discontinuity exists in the variation of O.D. (at 2590 Å) with concentration of DNA in the absence of added electrolyte[1,4,10,11]. As it is probable that this phenomenon arises from the same process that is responsible for the discontinuity in the conductivity measurements, the effect of thermal denaturation of the O.D. of DNA was also investigated. It was found that the thermal treatment described above completely removed the discontinuity observed in the dependence of the u.v. absorption on concentration. At all concentrations up to $8 \cdot 10^{-4}$ *M* DNA the extinction coefficient of the thermally denatured solutions was constant at 8500 (measured in the absence of salt at room temperature) and identical with that of an initially native solution below the critical concentration zone. Again this would indicate that denaturation by dilution is responsible for the critical concentration phenomenon.

Light scattering investigations[4] have shown that aqueous DNA solutions at low concentration are denatured and display a decreased root-mean-square end-to-end distance at constant molecular weight. In view of this finding, and that presented in the present investigation, it appears that denaturation by dilution can explain satisfactorily the critical concentration phenomenon.

Sedimentation studies

Since it would appear that the critical concentration is produced by denaturation of the DNA, it would be expected that two boundaries would be observed in sedimentation velocity experiments at DNA concentrations within the critical zone where both native and denatured DNA coexist. The presence of both native and denatured DNA together would be expected from the known heterogeneity of calf thymus DNA. Differences in sedimentation velocity would be expected, as the native species is relatively rigid, while denatured DNA, although it has the same molecular weight, would not necessarily be restrained to the dimensions imposed on the native structure.

An investigation of the sedimentation of DNA solutions at concentrations within the critical concentration zone has shown that the formation of two boundaries is very dependent upon the salt concentration of the solution being studied[12]. With solutions from which salt had been rigorously excluded and which have a DNA concentration of $2 \cdot 10^{-4}\,M$, sedimentation analysis shows (Fig. 6a) that, whilst no resolution into two distinct boundaries occurs, a diffuse leading edge is obtained in addition to a hypersharp trailing edge. This observation suggests the presence of more than one component. In 0.2 M sodium chloride, with the same DNA concentration, no further resolution was obtained, although the denatured material would still be present. At a sodium chloride concentration of $2.5 \cdot 10^{-4}\,M$, however, resolution into two boundaries was observed[12] (Fig. 6b). Although the reason why this resolution only occurs over a limited salt concentration is, at present, not known, the observation nevertheless confirms the theoretical prediction of more than one boundary. A study of the sedimentation velocity of fully denatured DNA suggests that the slower moving component corresponds to denatured DNA.

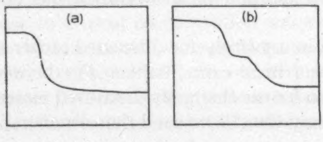

Fig. 6. Tracings of sedimentation velocity boundaries of DNA solutions. Tracings to be considered as inverted plots of DNA concentration.

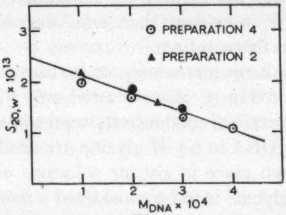

Fig. 7.

In the absence of salt, it was observed that no rapid change of the sedimentation coefficient occurred on dilution through the critical concentration zone. The values of $s_{20,w}$ (Fig. 7) show a linear relationship with concentration with no change in slope at the critical concentration. The values given in Fig. 7 were estimated from the half concentration point of the total asymmetric boundary.

ACKNOWLEDGEMENTS

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REFERENCES

[1] R. B. Inman and D. O. Jordan, *Biochim. Biophys. Acta*, 42 (1960) 421.
[2] R. Thomas, *Biochim. Biophys. Acta*, 14 (1954) 231.
[3] P. D. Lawley, *Biochim. Biophys. Acta*, 21 (1956) 481.
[4] L. F. Cavalieri, M. Rosoff and B. H. Rosenberg, *J. Am. Chem. Soc.*, 78 (1956) 5239.
[5] J. H. Coates and D. O. Jordan, *Biochim. Biophys. Acta*, 35 (1959) 309.
[6] R. A. Robinson and R. H. Stokes, *Electrolyte Solutions*, Chapter 6, Butterworths Scientific Publications, 1955.
[7] E. P. Geiduschek and I. Gray, *J. Am. Chem. Soc.*, 78 (1956) 879.
[8] S. Rice and P. Doty, *J. Am. Chem. Soc.*, 79 (1957) 3937.
[9] P. Doty and S. Rice, *Biochim. Biophys. Acta*, 16 (1955) 446.
[10] J. Pouyet, G. Schiebling and H. Schwander, *J. chim. Phys.*, 47 (1950) 716.
[11] M. Dhaussy, *J. chim. Phys.*, 52 (1955) 150.
[12] R. B. Inman and D. O. Jordan, *Biochim. Biophys. Acta*, 37 (1960) 162.

RIBONUCLEIC ACID FROM *ESCHERICHIA COLI*: ELECTRON MICROSCOPICAL STUDY

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SUMMARY

The dimensions of high-molecular weight RNA from *E. coli* were measured by electron microscopical methods. Filaments 10 to 13 Å in diameter, and 2000–4000 Å in length, could be observed in preparations obtained from salt-free aqueous solutions of RNA. When ammonium acetate solutions were used, individual fibers became rare, and granules and various forms of aggregates dominated the picture.

INTRODUCTION

RNA from *E. coli* has been previously characterized by viscometry, streaming birefringence and potentiometry[1,2]. These studies indicated that this high molecular weight RNA is much more flexible in solution than DNA, as would be in keeping with a single stranded structure.

In the present study an attempt has been made to determine the dimensions of *E. coli* RNA molecules, using electron microscopical methods.

Electron micrographs of TMV RNA have been prepared by SHUSTER *et al.*[3] and HALL[4], using an improved technique for visualizing macromolecules, has compared electron micrographs of calf liver and TMV RNA and salmon sperm DNA.

MATERIALS AND METHODS

RNA from *E. coli* was prepared as previously described[2]. A suspension of "protoplasts" was extracted with a phenol–water mixture, the RNA was precipitated by ammonium sulfate, dialyzed against dilute sodium chloride solution, and then lyophilized. The RNA revealed two boundaries in the ultracentrifuge, having sedimentation constants of 16.5 and 23.7 S (as determined using the u.v. absorption optical system). RNA was dissolved in distilled water (1 mg/ml). Complete solution was obtained after 30 to 60 min. Aliquots were diluted with water or with 0.1 *M* ammonium acetate (pH 6.8) to a final concentration of 0.1 mg/ml.

DNA from T_2 bacteriophage was prepared by osmotically shocking a phage suspension. The bacteriophage particles were suspended in 3 *M* ammonium acetate

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Abbreviations: DNA, deoxyribonucleic acid; RNAase, ribonuclease; RNA, ribonucleic acid; TMV, tobacco mosaic virus.

($2 \cdot 10^{11}$ particles/ml) and then diluted fifty fold with 0.1 M ammonium acetate. The suspension was clarified by centrifugation for 40 min at 105,000 $\times g$.

Crystalline pancreatic RNAase was an Armour and Company product.

Electron microscopy

Freshly cleaved mica was cut into rectangles of the size of a microscope slide. The mica rectangles were dipped in a 0.5 % parladion (Mallinckrodt Chemical Works) solution in redistilled amyl acetate. After drying at room temperature in an erect position under a cover, the film from the freshly-cleaved mica surface was floated onto glass doubly-distilled water. The grids were deposited on the floating film, and taken up on a glass slide[5]. Solutions were spread as fine droplets onto the film covering the grids on the slide, using, as the case may be, laboratory made low and high velocity spray guns. The preparations were dried at room temperature in a slightly open petri dish. In some experiments, a drop of the solution was deposited on the film on the grid, the preparation frozen on a copper block previously immersed in liquid-air and freeze dried in a Virtis apparatus.

The dried preparations were shadow-cast with either a chromium–nickel alloy, or platinum at shadow to height ratios 6:1 to 10:1. The films were backed with a thin supporting layer of SiO (see ref. 6). The platinum was evaporated from a tungsten spiral[7]. An aqueous suspension of polystyrene spheres of 0.34 μ diameter was added to the solution, to aid in the location of the droplets and in the exact determination of the shadow-casting angle and the direction of shadow. An RCA EMU 2A Electron Microscope was used with a locally made improved specimen stage, with a 50 μ objective aperture and with apertures at the intermediate and condenser lenses.

RESULTS AND DISCUSSION

Serious difficulties were encountered in stripping off the film from the mica surface, if the shadow casting and reinforcement with SiO were done directly on the mica as described by Hall. This was overcome by floating the collodion film first and then spraying the sample onto the surface of the film which had been in contact with the mica. This surface, being a true replica of the smooth surface of the mica, was structureless and suitable for the purpose intended. The shadow casting and reinforcing with SiO were done directly on the film.

Chromium–nickel alloy was used in some experiments because better contrast was attained with this alloy than with platinum. The larger grain, however, limited its use for more precise measurements. For this purpose platinum had to be employed.

Hydrodynamic measurements in solution have indicated that RNA molecules fold up into more compact structures when salt is added to the solution[2]. An attempt was therefore made to visualize RNA in both the more compact and the more unfolded state by this means and indeed the electron micrographs of RNA obtained from water solution showed molecules in the form of threads. A strong tendency for aggregation was noticed, especially at the center and border of the micro drops, probably due to the surface tension of the drying droplets. Using the high pressure gun single threads, 10–13 Å in diameter and 200–2000 Å in length (with a maximum in the distribution at between 200–600 Å), were generally found outside the periphery of the droplet (Fig. 1). Using the low pressure gun, the average length was higher (1200–4000 Å).

Fig. 1. *E. coli* RNA in water solution sprayed from a high pressure gun and air-dried at room temperature. Shadow cast with Pt at a shadow to height ratio of 6:1. S, direction of the shadow; D, border of the micro-drop; G, granule; F, fiber.

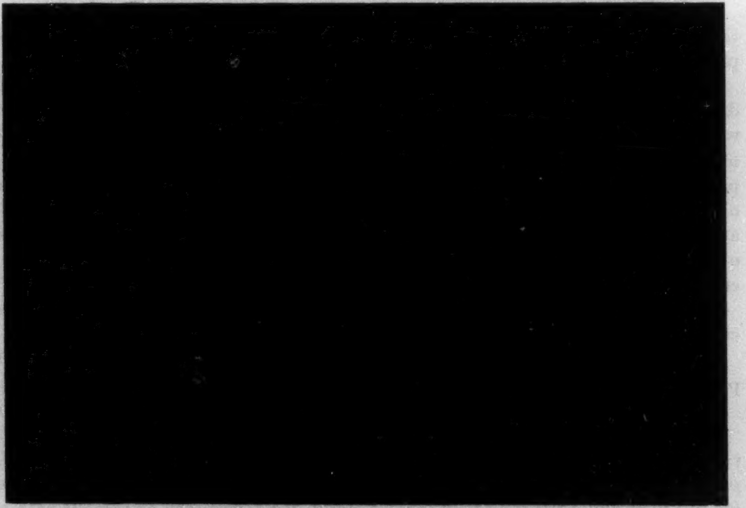

Fig. 2. *E. coli* RNA in water solution sprayed from low pressure gun and air-dried at room temperature. Shadow cast with Pt at a shadow to height ratio of 6:1.

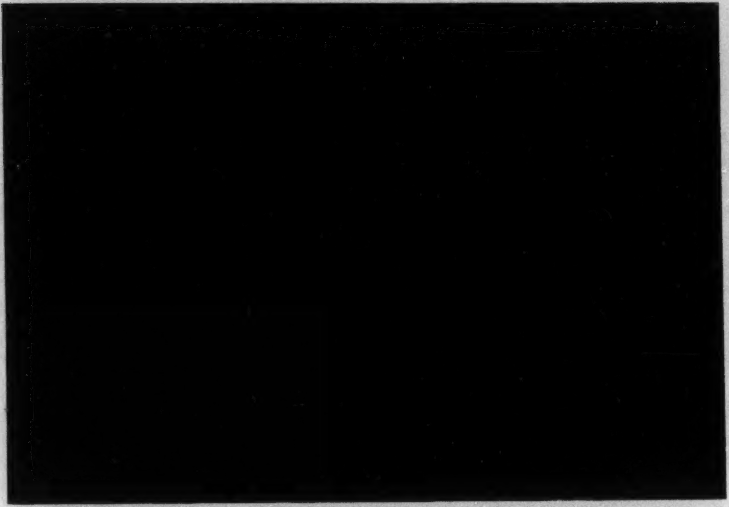

Fig. 3. *E. coli* RNA in 0.1 *M* ammonium acetate solution sprayed from a high pressure gun and air-dried at room temperature. Shadow cast with Pt at a shadow to height ratio of 10:1.

The same diameter values were observed (Fig. 2). Occasionally small flat granules, 400–600 Å in diameter, were noticed.

When RNA deposited from ammonium acetate solution was examined, individual threads became rare while granules and various forms of aggregates dominated the picture (Fig. 3).

In freeze-dried preparations, the material was found to be more homogeneously dispersed on the surface of the film. In preparations made by this technique from water solutions individual fibers were much more difficult to locate, due to the absence of microdroplets. The filaments measured from 1500–4000 Å in length (Fig. 4). A particular form of aggregation was revealed in these preparations that was not observed when the material was sprayed and air-dried at room temperature (Figs. 4 and 5). This aggregation was both longitudinal and lateral and showed a high degree of organization. The latter may be attributed to crystallization phenomena, apparently due to imperfections in the freeze-drying technique.

No difference in the form of the molecules, or in the nature of this aggregation could be revealed by varying the pH of the solution from 6 to 9.

When RNAase was introduced into the test tube containing the RNA, prior to preparation of the specimen, neither filaments nor granules could be detected.

DNA fibers appeared as long smooth strands, about 20 Å in diameter (Fig. 6).

The values 10–13 Å found for the RNA diameter are lower than the 15 Å estimated by SHUSTER *et al.*[3] for the finest fibers they could observe in TMV RNA preparations. They are also considerably lower than the values published by HALL for calf liver and TMV RNA[4]. In HALL's study, RNA was seen as threads with an apparent thickness of about 30 Å. According to HALL the molecules were thicker

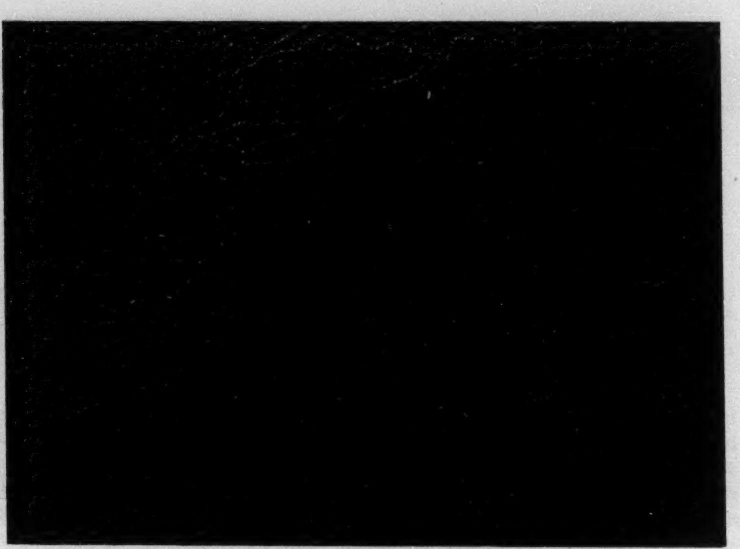

Fig. 4.

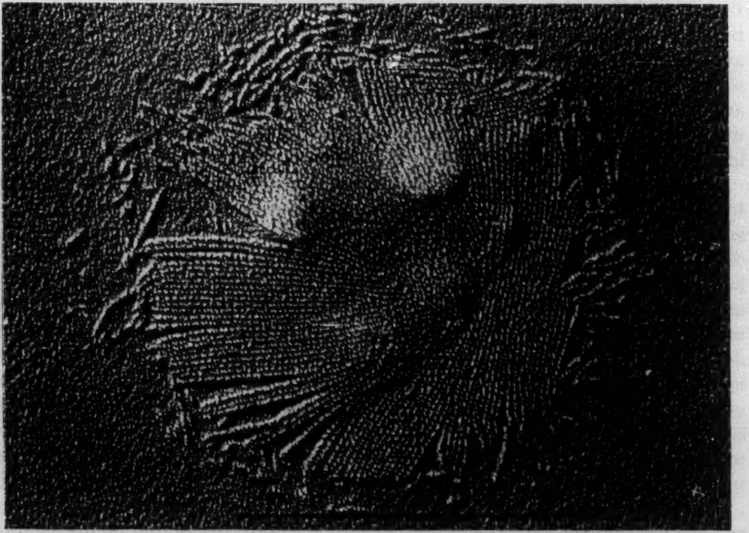

Fig. 5.

Figs. 4 and 5. *E. coli* RNA freeze-dried and shadow cast with chromium–nickel alloy at a shadow to height ratio of 6:1.

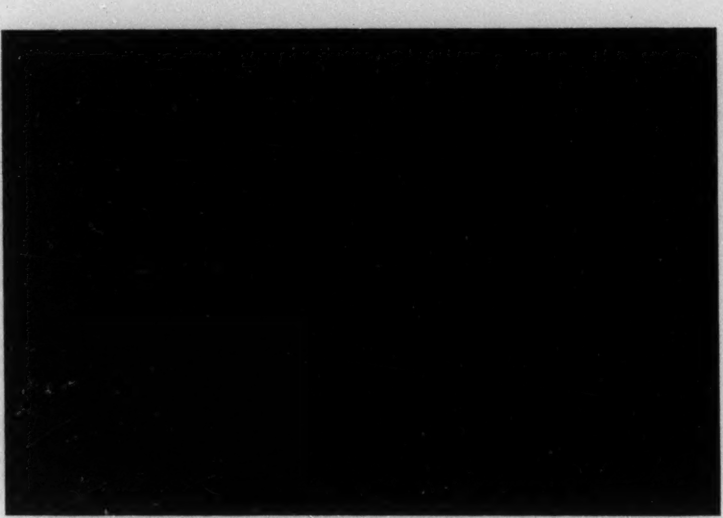

Fig. 6. Bacteriophage T_2 DNA in 0.1 *M* ammonium acetate solution sprayed from a low pressure gun and air-dried at room temperature. Shadow cast with Pt at a shadow to height ratio of 6:1.

than expected, and he thought that they were "probably partially coiled up on themselves". The present result for *E. coli* RNA would indicate that these molecules are being observed practically unfolded, probably due to the fact that aqueous solutions were used. Whenever salt solutions were used in our study granules appeared and practically no fine filaments were observed. These findings are in accordance with information derived from the hydrodynamic measurements[2], and support the idea that RNA is a molecule capable of great degree of coiling.

The RNA chain of *E. coli* appeared to be granular in nature, in contradistinction to the smooth and rigid DNA strands. This granular character of the fibers made it somewhat difficult to distinguish between a single long fiber and shorter ones, longitudinally aggregated. However, the majority of the fibers measured were found to be between 2000 and 4000 Å when the low pressure gun was used for spraying, or when using the "freeze-drying" technique. When the high pressure gun was used, the majority of the fibers were between 200 and 600 Å, and were apparently broken down by the treatment. This was confirmed by an ultracentrifuge and viscosity investigation of an RNA solution before and after passage through the high pressure gun. Fig. 7 shows the change in sedimentation pattern. The high molecular weight component has almost disappeared and a broad shoulder is in evidence at the low molecular weight end. The viscosity number η_{sp}/c fell from 1400 to 590 ml/g after spraying (viscosity measured at a concentration of $1.3 \cdot 10^{-4}$ g/ml in 10^{-3} *M* NaCl).

From the agreement between the pictures obtained by the freeze-drying technique and the low-pressure spray technique there is reason to believe that no degradation of the RNA molecules took place. Having regard to the high molecular weight of the molecules the strand lengths observed are much too short if considered as fully

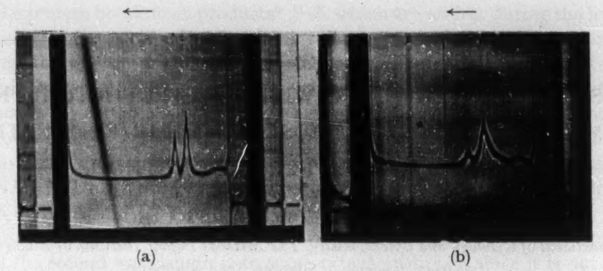
(a) (b)

Fig. 7. Effect of spraying on the sedimentation diagram of *E. coli* RNA. (a) RNA solution in 0.2 *M* NaCl. Picture taken at bar angle of 60° after 16 min at 52,640 rev./min and 23°. RNA concentration about 5 mg/ml. (b) RNA solution in 0.2 *M* NaCl after spraying from high pressure gun. Picture taken at bar angle of 60°, after 16 min at 56,000 rev./min and 21°. RNA concentration about 5 mg/ml.

stretched chains. Similarly the diameters of 10–13 Å are too large for an extended single chain. It is not unreasonable to suppose therefore that in the state visualized the single strand of RNA twisted itself up perhaps in helical fashion in which case both the diameter and length will be in agreement with the molecular weight. It is interesting to note that measurements of optical rotation[8], viscosity, sedimentation and potentiometric titration[9] have indicated the possibility of a high degree of secondary structure of *E. coli* RNA even in solution.

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REFERENCES

[1] H. Eisenberg and U. Z. Littauer, *Bull. Research Council Israel*, 7A (1958) 115.
[2] U. Z. Littauer and H. Eisenberg, *Biochim. Biophys. Acta*, 32 (1959) 320.
[3] H. Shuster, G. Schramm and W. Zillig, *Z. Naturforsch.*, *B* 11 (1956) 339.
[4] C. E. Hall, *Proc. IVth International Congress of Biochemistry*, Vol. 9, 1958, p. 90.
[5] C. E. Hall, *Introduction to Electron Microscopy*, McGraw-Hill Book Company, 1953, p. 315.
[6] C. E. Hall, *J. Biophys. Biochem. Cytol.*, 2 (1956) 625.
[7] H. Moor, *J. Ultrastructure Research*, 2 (1959) 393.
[8] R. A. Cox and U. Z. Littauer, *J. Mol. Biol.* in the press.
[9] R. A. Cox and U. Z. Littauer, *Nature*, 184 (1959) 818.

SUBSTANCES INTERFERING WITH SPECTROPHOTOMETRIC ESTIMATION OF NUCLEIC ACIDS AND THEIR ELIMINATION BY THE TWO-WAVELENGTH METHOD

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SUMMARY

The uniformity of the optical properties of substances interfering with the spectrophotometric determination of nucleic acids in different tissues has been proved. The absorption spectra of these substances show that the chief conflicting component is most probably tyrosine. The application of the two-wavelength method is therefore very convenient for nucleic acid estimations in animal tissues. The two wavelengths at which the optical density should be measured are 260 mμ and 286 mμ for RNA and 268 mμ and 284 mμ for DNA. A comparison of results obtained by different methods is presented.

INTRODUCTION

It is obvious from investigations made in recent years that in addition to the RNA and DNA fractions, nucleic acid extracts obtained by the Schmidt-Thannhauser procedure[1] contain numerous contaminants interfering with the nucleic acid determination by phosphorus analysis[2-4] and pentose colour reactions[3,5-9], as well as by ultraviolet absorption photometry[9-14]. The Ogur–Rosen method[15], by which purer extracts are obtained, does not, however, ensure the complete absence of interfering substances[6,9,16] and, what is more important, leads to an incomplete separation of RNA from DNA[6,17-21]. In order to avoid the interfering substances, different techniques have been used: reducing the time and lowering the temperature of the alkaline hydrolysis[11]; eliminating the interfering substances by adsorption on Dowex[9], by paper electrophoresis[2] or by paper chromatography[13]; fractionating nucleoproteins to a purer state prior to their determination[22]. The first of these procedures ensures neither the complete elimination of the contaminants nor the complete extraction of RNA[23]. The remaining procedures are too laborious for serial investigations and are not convenient as micromethods.

Taking into consideration the advantages of the spectrophotometric determinations of nucleic acids, a number of authors have tried to apply this method for quantitative nucleic acid determination in tissue fractions[3,15,20,21,24,27], although interfering substances have always been detected. The main contaminants are supposed to be protein breakdown products[9–11, 29], which are set free during the hydrolytic separation of nucleic acids. Until now no suitable methods have been proposed for calculating the nucleic acid content by measuring the optical density of the contaminated fractions[14, 29, 30]. The unknown specific extinction coefficient of the contaminants for suitable wavelengths has made some authors conclude that such a calculation would be impossible[10]. In order to eliminate the cell-protein absorption, a two-wavelength method was introduced twenty years ago for cytochemical estimation of NA[31, 32]. Until now such a method has not been used for quantitative determination of RNA and DNA in tissue extracts. In the few investigations in which the difference in the absorbance at two wavelengths is used[21, 27, 33, 34], the appropriate choice of the second wavelength is to some extent arbitrary, since it is not based on the spectral characteristics of the absorbing contaminants.

In this paper we demonstrate the uniformity of the optical properties of the contaminants in different animal tissues. This makes it possible to determine suitable wavelengths at which the absorption of the contaminants would be eliminated by the two-wavelength method.

Prior to the application of the ΔE method it was necessary to prove the additivity of the optical density E_λ of a mixture containing an amount N of nucleic acid with absorptivity k_λ and an amount X of contaminants with absorptivity k'_λ, *i.e.* that

$$E\lambda = k\lambda N + k\lambda X \tag{1}$$

This additivity is admitted generally[34]; but there are data in the literature showing that the absorptivity of a mixture containing adenylic acid and tyrosine is far from being additive[35]. In our experiments, however, we have shown that the optical density of a mixture of pure hydrolysed RNA (or DNA) and protein breakdown products (both in N PCA) is strictly additive[12]. Having proved the validity of (1) in such a way, one can estimate the quantity of nucleic acids by measuring the optical density of the extracts at two wavelengths at which the absorption coefficients of the contaminants are equal. It is convenient to fix the first wavelength at the absorption maximum of nucleic acids. Denoting the second wavelength as λ_0, the amount of nucleic acid according to (1) will be

$$N = \frac{E\lambda_{\max} - E\lambda_0}{k\lambda_{\max} - k\lambda_0} = K\Delta E \tag{2}$$

The difficulties in determining λ_0 are connected with finding an appropriate absorbing standard. In our investigations it was possible to determine λ_0 from the absorption spectra of the tissue-proteins breakdown products and to show the constancy of this wavelength for most of the normal and malignant tissues investigated.

METHODS

Material

Various normal and malignant animal tissues were obtained in the following way: 1. *Livers* of albino rats, albino mice, frogs (*R. ridibunda*) and tritons (*Triturus cristatus*) were removed immediately after killing the animals by decapitation. 2. *Skin* from the back of albino mice, frogs and tritons was used. The depilation of the mouse skin was performed with a mixture of wax and colophony. 3. *Muscles* from the thigh of albino mice, frogs and tritons were used. 4. *Epidermis* from the back of albino mice was separated from the dermis according to our method using subcutaneous injection of 2% ice-cold citric acid[36]. 5. *Malignant tumors*: 15-day-old transplantable Guérin carcinoma, Ehrlich ascites carcinoma and benzpyrene-induced skin carcinoma in mice were investigated. Care was taken to eliminate the necrotic parts of the solid tumors. Immediately after removal the ascites fluid was diluted with 100 vol. of ice-cold Krebs–Ringer solution, then centrifuged. 6. *Actinia equina* from the Black Sea was used *in toto* after 30 days of fasting.

Nucleic acids determination

In some of the tissues (rat liver, mouse skin, Guérin carcinoma) the results obtained for the nucleic acid content, determined by phosphorus analysis, pentose reactions and ultraviolet absorption were compared. It was therefore necessary to treat this material according to the classical procedure as follows.

A

(a) *Preliminary treatment.* 1. The weighed material is homogenized in a glass homogenizer with 10% ice-cold TCA. 2. The homogenate is washed twice with 5% ice-cold TCA and once with 96% alcohol. 3. It is then boiled in a water bath for 3 min with alcohol–ether (3:1) and for 30 min with methylalcohol–ether (1:1). 4. It is washed with ether and dried at 37°.

(b) *RNA-fraction.* 1. The dry residue is incubated for 18 h in *N* KOH at 37° (1 ml KOH to each 100 mg fresh tissue). 2. Conc. PCA (0.1 ml to each ml KOH) is added to the ice-cold hydrolysate. 3. The precipitate is centrifuged and washed twice with ice-cold *N* PCA. 4. Supernatant and washings are mixed together and made up to a given volume with *N* PCA.

(c) *DNA-fraction.* The precipitate from (b) is extracted twice according to Ogur–Rosen with *N* PCA, each time for 30 min, at 80° and the two extracts are combined.

When the nucleic acid content is determined only by the ultraviolet absorption the careful extraction of lipids is not necessary. It is also preferable to estimate the amount of nucleic acids on dry weight and to have the possibility of storing the material at some steps of the procedure. In this case the following technique (allowing considerable reduction of the quantity of material needed) was used.

B

(a) *Preliminary treatment.* 1. The material is frozen and cut in 25 μ slices with the freezing microtome. 2. The slices are put in ice-cold absolute alcohol and washed once more in absolute alcohol (if necessary they can be stored in the second alcohol). 3. They are washed twice with ether for 30 min and dried at 37° overnight. The dried material can be stored in a dessicator. 4. A weighed quantity of the material is treated twice with ice-cold 0.2 *N* PCA for 15 min each time (the simplest technique is first to add some drops of 0.2 *N* PCA to the dried residue, stirring it with a glass rod). 5. After washing with 96° alcohol and with ether it is dried at 37°.

The following procedure is like Ab and Ac.

Phosphorus determination was carried out according to KUTTNER AND COHEN[37].

The orcinol reaction was performed according to MEYBAUM[38], with a 45-min heating period[39]. The absorbance was determined by means of a Pulfrich photometer at S_{66}. The data were expressed in phosphorus following the relation $P = \frac{E + 0.045}{0.1250}$.

The deoxyribose was determined by the diphenylamine reaction according to DISCHE[40]. The absorbance was measured with a Pulfrich photometer at S_{61} and the P content calculated by the formula $P = E/0.0195$.

The optical density in the ultraviolet was determined with the Zeiss universal spectrophotometer. The nucleic acid content was expressed in mg of phosphorus on the basis of $\varepsilon(P)_{RNA} = 10130$ and $\varepsilon(P)_{DNA} = 8782$.

Tissue breakdown products

The appropriate absorbing standards were obtained from the tissues in the following way:

1. The material is treated as in Aa or Ba 1–3.
2. The nucleic acids and acid-soluble compounds are extracted simultaneously with *N* PCA at 80° six times, for 15 min each. The complete extraction of absorbing material was controlled spectrophotometrically.
3. The protein residue is washed with distilled water, alcohol, ether, and dried at 37°.
4. The dried material is incubated in *N* KOH for 18 h at 37° or is kept for 1 h in a boiling water-bath, the two procedures giving breakdown products with the same spectra, differing only in the quantity of absorbing substances released.
5. The hydrolysate is cooled and the proteins precipitated with conc. PCA as in Ab 2–4. The mixed supernatant and washings are brought to a given volume and used for spectrophotometry.

In all spectrophotometric measurements of RNA fractions and tissue breakdown products, blanks are used of *N* KOH treated with conc. PCA, centrifuged and diluted with *N* PCA to the same volume as the investigated solution. DNA fractions are measured against *N* PCA.

RESULTS AND DISCUSSION

Optical characteristics of the interfering substances

The absorption spectra of the tissue breakdown products are presented on Fig. 1. In spite of the very different origin of the tissues investigated, these spectra differ mainly in the quantity of absorbing material per 100 g of tissue, having the same spectral characteristics—maximum at 275–278 mμ and minimum at 257–260 mμ. For the ΔE method it is necessary to determine the appropriate wavelength λ_0 at which the absorbance is equal to the absorbance at 260 mμ, for the RNA estimation, and at 268 mμ for the DNA estimation. Under the above conditions of alkaline hydrolysis it was found that all investigated tissues of vertebrates (excluding the frog skin) give breakdown products with a $\lambda_0 = 286$ mμ for RNA and $\lambda_0 = 284$ mμ for DNA. In the frog skin the presence of substances with higher absorption at 260 mμ is detected in the tissue hydrolysates. In this material therefore the λ_0 is shifted to 283 mμ for RNA and to 280 mμ for DNA.

The comparison of the absorption curves of the tissue breakdown products with the spectrum of tyrosine (Fig. 1) makes it seem probable that the main absorbing component of the conflicting substances in the Schmidt–Thannhauser alkaline hydrolysates is this aromatic amino acid. The presence of tyrosine and probably tryptophan in these hydrolysates has already been suggested by MAURITZEN *et al.*[10].

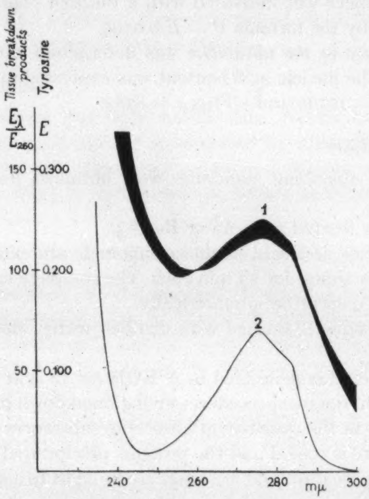


Fig. 1. 1. Absorption spectra of tissue-protein breakdown products from liver (rat, frog, triton), skin (mouse, triton), epidermis (mouse), muscle (mouse, frog, triton) and malignant tumors (Guérin carcinoma, Ehrlich ascites carcinoma and benzpyrene-induced skin carcinoma)—all in normal perchloric acid. Data expressed in percentages of absorbance at 260 mμ. 2. Absorption spectrum of 10^{-4} *M* solution of DL-tyrosine in normal perchloric acid.

The obligatory presence of these amino acids in all animal tissue proteins explains the uniformity of the absorption spectra of the interfering substances released after alkaline hydrolysis of quite different tissues. In this connection some data indicating the similarity of amino acid composition of microsomal nucleoprotein particles should be mentioned[41]. The constancy of λ_0 in different tissues may be explained by taking into account the absorption spectrum of tyrosine as a chief component of the conflicting substances. In *N* PCA the absorptivity of tyrosine at 260 mμ is equal to the absorptivity at 287.5 mμ and the absorptivity at 268 mμ equals the absorptivity at 284.5 mμ. The presence of other substances absorbing to the left of the tyrosine maximum explains the shifting of λ_0 in the different animal tissues to 286 mμ for RNA and to 284 mμ for the DNA.

Elimination of the interfering substances by the two-wavelength method

It is obvious from the above data that the ΔE method is applicable to various animal tissues owing to the identical absorption spectra of their tissue-protein breakdown products. Using equation (2) the quantity of RNA and DNA in mg of phosphorus per 100 g of tissue weight will be:

$$\text{RNA} = K_{\text{RNA}} \frac{(E_{260} - E\lambda_0)\,V}{w.l} \text{ mg per cent P} \qquad (3)$$

$$\text{DNA} = K_{\text{DNA}} \frac{(E_{268} - E\lambda_0)\,V}{w.l} \text{ mg per cent P} \qquad (4)$$

V being the volume of the extract in ml, w the weight of the investigated material in mg, l the length of the light path in cm. The coefficient K depends on the second wavelength λ_0 as follows

$$K = \frac{M}{\varepsilon(\text{P})} \cdot \frac{r}{(r - 1)} \qquad (5)$$

where M is the molecular weight of phosphorus, ε(P) the molecular extinction coefficient of nucleic acid per g-atom of phosphorus, r the ratio of the absorbance of pure nucleic acid at 260 mμ (resp. 268 mμ) to the absorbance at λ_0.

On the basis of the above cited values for λ_0 and ε(P), $r = E_{260}/E_{286}$ being 2.2 for RNA and $r = E_{268}/E_{284}$ being 1.79 for DNA ,the coefficient K_{RNA} is 561 and K_{DNA} is 800. Since λ_0 may be different in some cases, the coefficients should be calculated according to (5). Within certain limits of λ_0 the dependence of K on λ_0 is almost linear and K may be determined with an error smaller than 1% from the equations

$$K_{\text{RNA}} = 9341.2 - 30\,\lambda_0 \text{ (for } \lambda_0 \text{ within the limits 284–289 m}\mu\text{)} \qquad (6)$$

$$K_{\text{DNA}} = 15284 - 51\,\lambda_0\text{; (for } \lambda_0 \text{ within the limits 283–287 m}\mu\text{)} \qquad (7$$

Determination of nucleic acid content by estimation of their various components

In Table I are shown the different values obtained by the determination of the nucleic acids by estimation of their various components. Highest values for RNA are obtained by the orcinol reaction and by ultraviolet photometry when absorption at 260 mμ is used. Although the values obtained in these two ways are in good agreement with most of the data in the literature[6, 42, 43] they must be considered as being significantly increased on account of the interfering substances. Despite the fact that such substances are known both for the orcinol reaction and for ultraviolet absorption, these two techniques are nevertheless widely employed in nucleic acid determination. The 15 different tissues we have investigated show the ubiquitous presence of u.v.-absorbing material other than nucleic acids, both in the RNA and in the DNA fractions (Fig. 2 and 3). The main difference between the spectra of the pure nucleic acids and the tissue nucleic acids extracts lies in the very strong absorption of the latter at the shorter wavelengths (below 240 mμ) and in their higher absorption at 270-290 mμ. In this respect our results are in good agreement with the recent data of DE DEKEN *et al.*[9].

The estimation of RNA by direct phosphorus analysis gives lower results. Taking into account the established presence of non-nucleotidic phosphorus in the RNA fraction of Schmidt–Thannhauser[2–4], we must consider these values as overrated too.

The RNA content determined by the ΔE method is considerably lower than that estimated by any other procedure. The direct phosphorus analysis gives RNA values 31% (in the rat liver), 22% (in the Guérin carcinoma) and 18% (in the mouse

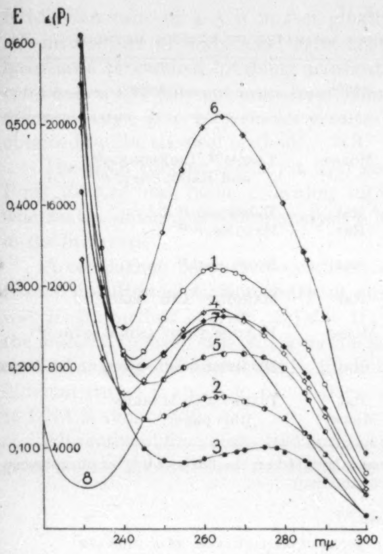
TABLE I

DETERMINATION OF NUCLEIC ACID CONTENT OBTAINED BY ESTIMATION OF THEIR VARIOUS COMPONENTS

All data are expressed as mg of phosphorus/100 g of fresh tissue.

Tissue	Number of cases		RNA				DNA				RNA/DNA			
			ΔE	E_{260}	*Ribose*	*P*	*ΔE*	E_{268}	*Deoxyribose*	*P*	*ΔE*	E_{max}	*Pentose*	*P*
Rat liver	5	M	54.4	98.3	99.1	80.0	29.4	38.1	—	18.5	1.86	2.58	—	4.32
		m	± 1.3	± 2.5	± 4.9	± 3.3	± 1.6	± 1.8		± 0.6				
		per cent	100	181	182	147	100	131		63				
	5	M	51.6	92.2	87.4	74.1	22.0	30.5	20.6	14.0	2.37	3.02	4.24	5.29
		m	± 1.5	± 2.1	± 2.1	± 1.9	± 1.0	± 1.8	± 1.3	± 2.0				
		per cent	100	179	169	143	100	139	94	64				
Mouse skin	5	M	19.2	39.2	38.4	—	27.1	38.5	27.5	—	0.71	1.02	1.40	—
		m	± 1.5	± 1.8	± 2.5		± 2.4	± 3.4	± 1.9					
		per cent	100	204	200		100	142	101					
	5	M	17.1	34.4	33.8	21.8	24.1	31.1	26.0	22.7	0.71	1.10	1.03	0.96
		m	± 1.3	± 2.1	± 2.2	± 1.5	± 1.3	± 1.4	± 1.5	± 1.7				
		per cent	100	201	188	127	100	129	108	94				
Guérin carcinoma	1		51.9	82.7	90.6	63.6	88.2	108.5	91.7	80.3	0.59	0.76	1.14	0.79
		per cent	100	159	175	123	100	123	104	91				

M – mean.
m – standard error of the mean.



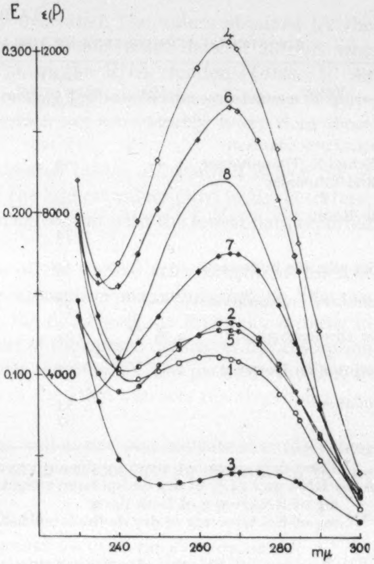


Fig. 2. Absorption spectra of RNA fractions obtained by the Schmidt–Thannhauser procedure and of pure yeast RNA alkaline hydrolysate—all in normal perchloric acid. E = optical density per 100 mg of fresh tissue (1, rat liver; 2, mouse skin; 3, mouse muscle; 4, Guérin carcinoma) or per 20 mg of dry de-lipidated tissue (5, epidermis of mouse skin; 6, Ehrlich ascites carcinoma; 7, benzpyrene-induced skin carcinoma). 8, ε(P) = molar extinction coefficient per g-atom of phosphorus of yeast RNA.

Fig. 3. Absorption spectra of DNA fractions obtained by hot perchloric acid extraction, compared with pure DNA treated in the same way. 1–7, as in Fig. 2; 8, DNA.

skin) higher than those obtained by the ΔE method. That is in good agreement with the data of DAVIDSON AND SMELLIE[2], who reported 25% non-nucleotidic phosphorus in the RNA fraction.

As far as DNA is concerned, the ΔE values are almost identical with the values obtained by the diphenylamine reaction. Taking into account the low sensitivity of the diphenylamine test, it is possible that the difference of 5–10% between the two methods is due to errors in the deoxyribose estimation. In this connection it is necessary to mention the note of DE DEKEN *et al.*[9] pointing out that lower results are obtained with the diphenylamine reaction when the DNA is extracted at a higher temperature and that a reduction of the deoxyribose content of DNA hydrolysate takes place when stored at room temperature.

It is worth noting that the direct phosphorus analysis of the DNA fraction always gives lower results for the DNA content than the values obtained by the ΔE method or by the diphenylamine reaction. The difference is especially great in rat liver where the direct analysis of the phosphorus leads to values 36%, and 32% lower than the ΔE and the diphenylamine results, respectively. This difference between the P analysis and the diphenylamine test is in agreement with the data

TABLE II

RIBONUCLEIC ACID CONTENT OF THE LIVER ESTIMATED BY VARIOUS METHODS

Method	*RNA*	*RNA-P mg per 100 g of fresh tissue**	*Animal*	*Author*
Classical procedures of Schmidt–Thannhauser and Schneider	—	77–100 93	Rat Mouse	Leslie[42], Tshepinoga[43] and Badolato *et al.*[6]
Ogur–Rosen	—	57	Rat	Badolato *et al.*[6]
	—	74	Rat	Hadjiolov[44]
Milder alkaline hydrolysis	556**	50	Rat	Scott *et al.*[11]
Preliminary precipitation	—	58	Rat	Manoïlov and Orlov[22]
Paper chromatography	2.98***	64	Mouse	Kleinschmidt and Manthey[13]
Adsorption on Dowex-2	2.63***	57	Rat	De Deken-Grenson and De Deken[9]
ΔE method		53 67	Rat Mouse	Tsanev and Markov (this paper)

* These data when not given by the authors are calculated on the basis of 9% of phosphorus in the RNA and 24% of dry de-lipidated weight of the liver.

** mg of RNA/100 g of fresh tissue.

*** mg of RNA/100 mg of dry de-lipidated tissue.

of Badolato *et al.*[6], who also report that the values for DNA obtained by the phosphorus determination are 17–31% lower than those obtained by the diphenylamine reaction. It has already been pointed out that the Ogur–Rosen method, avoiding the alkaline hydrolysis, gives significantly higher results for DNA than the Schmidt–Thannhauser procedure[6,44,45]. It would seem that this discrepancy is mainly due to incomplete extraction of RNA by the cold *N* PCA. It should be noted, however, that some authors admit an incomplete precipitation of DNA in the Schmidt–Thannhauser method[9,10,46,47]. Kleinschmidt and Manthey[13], using a chromatographic separation of nucleic acid compounds prior to their spectrophotometric determination, report, however, that a single hydrolysis of the tissue with *N* KOH does not result in any loss of DNA. Badolato *et al.*[6] report also that they never found diphenylamine-positive material in the RNA fraction. Our results, as well as the results of Badolato *et al.*[6] (higher values by the u.v. absorption and the diphenylamine test than by phosphorus analysis) suggest a partial dephosphorylation of DNA during the alkaline hydrolysis in which part of the DNA phosphorus is transferred to the RNA fraction. It is necessary to point out that the excess of phosphorus in the RNA fraction is always greater or equal to the missing phosphorus in the DNA fraction.

It would be of use to compare the values we have obtained for the nucleic acid content in rat liver with the numerous literature data concerning the nucleic acids in this organ. Most authors who have investigated the nucleic acids in rat and mouse liver by the Schmidt–Thannhauser procedure report data within 77–100 mg of P for RNA and within 16–26 mg of P for DNA per 100 g of fresh tissue[6,42,43]. It is obvious that these values can be considered as higher for RNA and slightly lower for DNA than the real ones. Therefore, according to these data, the accepted

RNA/DNA ratio of 4–5 is in fact greatly overrated. The values obtained by the *ΔE* method are in fairly good agreement with some recent data of authors who have used procedures involving purification of the RNA fraction (Table II). As can be seen, five different procedures, eliminating the interfering substances in quite different ways, give very similar results, which are considerably lower than those obtained by the classical methods.

The DNA content of the rat liver estimated by the *ΔE* method is 25.7 mg of P per 100 g of fresh tissue, coinciding with the highest values cited in the literature, whereas the direct phosphorus analysis is in agreement with the lowest data reported in the literature.

A comparison between the estimation of the nucleic acid content in different tissues by ultraviolet photometry at the absorption maximum and by the two wavelength-method is given in Table III; the differences are especially striking in the muscles. In every case only a certain part of the optical density at the absorption maximum is due to nucleic acids. Excluding the muscles, the part due to RNA in different tissues is about 50–60%, whereas in the DNA extracts the absorbance due to DNA is about 75%.

All results of the above-cited authors as well as our own indicate that the values given in the literature for the RNA content in various tissues determined by the

TABLE III

NUCLEIC ACID ANALYSIS IN VARIOUS TISSUES BY ULTRAVIOLET PHOTOMETRY AT THE ABSORPTION MAXIMUM AND BY THE *ΔE* METHOD

All data are expressed as mg of phosphorus/100 g of fresh tissue or of dry de-lipidated tissue*.

Material	*Number of cases*	*RNA*			*DNA*			*RNA/DNA*	
		E_{260}	ΔE	$\Delta E/E_{260}$	E_{268}	ΔE	$\Delta E/E_{268}$	E_{max}	ΔE
Liver									
1. Rat	10	95.3	53.3	0.56	34.3	25.7	0.75	2.80	2.12
2. Frog	1	69.2	41.5	0.60	84.8	63.5	0.75	0.82	0.65
3. Triton	2	42.1	23.6	0.56	80.9	58.4	0.72	0.52	0.41
Skin									
4. Mouse	10	169.0*	84.0*	0.50	198.0*	142.0*	0.72	0.85	0.59
	10	36.8	18.1	0.49	34.8	25.6	0.74	1.06	0.71
5. Frog	2	31.0	18.3	0.59	24.6	13.0	0.53	1.26	1.41
6. Triton	2	51.3	30.5	0.60	59.1	42.2	0.72	0.87	0.74
Epidermis									
7. Mouse	5	351.0*	179.0*	0.51	241.8*	169.4*	0.70	1.45	1.05
Muscle									
8. Mouse	4	130.9*	29.2*	0.22	56.3*	20.8*	0.37	2.47	1.40
9. Frog	2	17.8	3.4	0.20	11.4	1.6	0.15	1.55	2.17
10. Triton	3	19.7	2.9	0.15	21.3	10.1	0.47	0.92	0.29
Malignant tumors									
11. Guérin carcinoma	1	82.7	51.9	0.63	108.5	88.2	0.81	0.76	0.59
12. Ehrlich ascites carcinoma	5	781.6*	491.3*	0.63	473.2*	344.9*	0.73	1.65	1.42
13. Benzpyrene-induced skin carcinoma	7	401.0*	211.0*	0.53	299.2*	213.9*	0.72	1.37	0.99
Actinia equina									
14. Total body	2	341.0*	190.0*	0.56	154.4*	91.6*	0.59	2.21	2.07

usually accepted Schmidt–Thannhauser technique are considerably higher than the real ones. It should be considered as obligatory in nucleic acid determinations to utilize only procedures that eliminate the interfering substances. In this respect the two-wavelengths method has all the advantages of the spectrophotometric methods, while avoiding at the same time the laborious purifying procedures.

REFERENCES

[1] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.*, 161 (1945) 83.

[2] J. N. Davidson and R. M. S. Smellie, *Biochem. J.*, 52 (1952) 599.

[3] J. E. Logan, W. A. Mannel and R. J. Rossiter, *Biochem. J.*, 51 (1952) 470.

[4] H. A. Deluca, R. J. Rossiter and K. P. Strickland, *Biochem. J.*, 55 (1953) 193.

[5] Z. Dische, in E. Chargaff and J. N. Davidson, *The Nucleic Acids, Chemistry and Biology*, Vol. I, Academic Press, Inc., New York, 1955.

[6] F. Badolato and A. Calabrese, *Ricerca sci.*, 25 (1955) 2558.

[7] T. F. Slater, *Biochim. Biophys. Acta*, 27 (1958) 201.

[8] F. J. Finnamore, *Exptl. Cell Research*, 12 (1957) 356.

[9] M. de Deken-Grenson and R. H. de Deken, *Biochim. Biophys. Acta*, 31 (1959) 195.

[10] C. M. Mauritzen, A. B. Roy and E. Stedman, *Proc. Roy. Soc.* (*London*), B 140 (1952) 18.

[11] J. F. Scott, A. P. Fraccastoro and E. B. Tafft, *J. Histochem. Cytochem.*, 4 (1956) 1.

[12] R. Tsanev and G. G. Markov, *Izvest. Biol. Inst. Bulg. Akad. Nauk.*, 10 (1960) 111 (Bulgarian).

[13] W. J. Kleinschmidt and J. A. Manthey, *Arch. Biochem. Biophys.*, 73 (1958) 52.

[14] J. Moulé, *Arch. sci. physiol.*, 7 (1953) 24.

[15] M. Ogur and G. Rosen, *Arch. Biochem.*, 25 (1950) 262.

[16] R. Tsanev and G. G. Markov, unpublished data.

[17] M. Ogur, S. Minkler, G. Lindegren and C. C. Lindegren, *Arch. Biochem. Biophys.*, 40 (1952) 175.

[18] E. K. Patterson and M. E. Dackermann, *Arch. Biochem. Biophys.*, 36 (1952) 97.

[19] V. A. Cassel, *J. Bacteriol.*, 59 (1950) 185.

[20] H. G. Loeb and E. Dickinson, *Arch. Biochem. Biophys.*, 57 (1955) 515.

[21] M. Teiichi, B. Wagner and B. Strauss, *Arch. Biochem. Biophys.*, 80 (1959) 442.

[22] S. E. Manoïlov and A. S. Orlov, *Biokhimiya*, 23 (1958) 662.

[23] R. Tsanev and G. G. Markov, unpublished data.

[24] K. K. Tsuboi, *Biochim. Biophys. Acta*, 6 (1950) 202.

[25] A. Ceriotti, *J. Biol. Chem.*, 198 (1952) 297.

[26] J. M. Webb, *J. Biol. Chem.*, 230 (1958) 1023.

[27] A. S. Spirin, *Biokhimiya*, 23 (1958) 656.

[28] J. Folch and F. N. Le Baron, *Federation Proc.*, 10 (1951) 185.

[29] E. Volkin and W. E. Cohn, in D. Glick, *Methods of Biochemical Analysis*, Vol. I, Interscience Publ. Inc., New York, 1954.

[30] J. M. Webb and H. B. Levy, in D. Glick, *Methods of Biochemical Analysis*, Vol. VI, Interscience Publ. Inc., New York, 1958.

[31] T. Caspersson and L. Santesson, *Acta Radiol.*, Suppl. 46, 1942.

[32] T. Caspersson, *Cell Growth and Cell Function*, Norton & Co. Inc., New York, 1950.

[33] J. I. Nurnberger, in R. C. Mellors, *Analytical Cytology*, McGraw-Hill Book Co., New York, 1955.

[34] O. Warburg and W. Christian, *Biochem. Z.*, 310 (1941) 384.

[35] A. A. Ferkhmin, *Dokl. Acad. Nauk S.S.S.R.*, 59 (1948) 945 (Russian).

[36] R. Tsanev and G. Markov, *Experientia*, (1960) in the press.

[37] T. Kuttner and H. R. Cohen, *J. Biol. Chem.*, 75 (1927) 517.

[38] W. Meybaum, *Z. physiol. Chem., Hoppe Seyler's*, 258 (1939) 117.

[39] H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.*, 167 (1947) 369.

[40] Z. Dische, *Mikrochemie*, 8 (1930) 4.

[41] P. O. Ts'O, J. Bonner and H. Dintzis, *Arch. Biochem. Biophys.*, 76 (1958) 225.

[42] I. Leslie, in E. Chargaff and J. N. Davidson, *The Nucleic Acids*, Chemistry and Biology, Vol. II, Academic Press Inc., New York, 1955.

[43] O. P. Tshepinoga, *The Nucleic Acids and Their Biological Role* (in Russian), Acad. Nauk Ukr. S.S.R., Kiev, 1956.

[44] A. A. Hadjiolov, *Z. Krebsforsch.*, 62 (1958) 361.

[45] T. K. Nikolov and P. K. Danev, *Ukrain. Biokhim. Zhur.*, 30 (1958) 652.

[46] C. J. Davidova, *Biokhimiya*, 19 (1954) 178.

[47] M. L. Drasher, *Science*, 118 (1953) 181.

THE EFFECT OF VARIOUS MONOVALENT ANIONS ON MYOSIN B SOLUTIONS

THE IDENTIFICATION OF ACTIN AS A PRODUCT OF ATP ACTION

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SUMMARY

Physical studies on Myosin B solutions in KSCN, KI, and in a variety of other ionic media reveal that exposure to these media leads to a dissociation into myosin and actin moieties. The actin appears in the globular form and may be clearly identified in the ultracentrifuge. The peak formed under these conditions (G-actin) is indistinguishable from the peaks formed in the same media by conventionally prepared actin or by redissolution of the pellet obtained by addition of ATP to Myosin B and strong centrifugation. It is concluded that actin exists in Myosin B in combination with myosin but otherwise in substantially the same form as is obtained by conventional procedures, employing treatment with non-aqueous solvents; and that actin is indeed a product produced by the action of ATP on Myosin B.

INTRODUCTION

Since the early work on the proteins of muscle it has usually been assumed that solutions obtained from 5 to 24-h extracts of striated muscle, after successive precipitations at low ionic strength, contain a complex of myosin and actin, which may be dissociated by the action of adenosine triphosphate (ATP)[*,1].

Doubts concerning this interpretation have persisted, however, and these have partly been based on the consistent failure of many workers to observe a separate actin component in, say, the ultracentrifuge upon addition of ATP[2-5]. In the ultracentrifuge 24-h preparations usually show a single, sharp, fast-moving ($S_{20,w} > 10S$) peak (Fig. 1). When ATP is added, a single, sharp peak with the sedimentation rate of myosin invariably becomes visible, but no component appears that is identifiable as actin, which under the usual conditions of pH ~ 7, in 0.6 M KCl, would presumably be in the fibrous form (F-actin), rather than in the globular form (G-actin). Proponents of the classical view insist that the F-actin released is of such low concentration, so polydisperse, and/or so fast-moving that it never appears in the Schlieren photograph[6]; but since F-actin prepared from extracts of muscle acetone powder is visible in the

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[*] We shall refer to these as "Myosin B" solutions and, in general, conform to the nomenclature suggested in ref. 4.

centrifuge, this argument is not entirely convincing and more definite proof is needed. Recently, several attempts have been made to obtain such proof. WEBER sedimented Myosin B solutions containing ATP in the preparative centrifuge and conclusively identified the supernatant as myosin by a variety of means[6]. However, it was only shown indirectly that the pellet contained actin by demonstrating that mixing a pellet extract with supernatant restored the super-precipitation property and the enzymic activity (measured in the presence of Mg^{++}), both characteristic of the original Myosin B solution. It has also been shown that the supernatant solutions do not reform the original fast component when the ATP has been enzymically destroyed[6], or when it is dialyzed out[5]. Thus it appears clear that a protein component essential to the formation of the characteristic particles in Myosin B can be separated from myosin by addition of ATP and strong centrifugation. A positive identification of this component as actin has not yet been made, however.

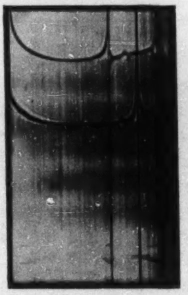

Fig. 1. Myosin B, 0.49%, centrifuged at 59,780 rev./min; bar angle, 60°; temperature, 11.9°; time, 32 min. Wedge cell, 0.6 M KCl, $S_{20,w} = 9.5$ S. Regular cell, 0.6 M KCl + $5 \cdot 10^{-3}$ M ATP, $S_{20,w} = 4.1$ S.

SZENT-GYÖRGYI was able to prepare actin from Myosin B by precipitating the myosin with alcohol[7], and GERGELY AND MARTONOSI prepared actin by making an acetone powder from a myosin B precipitate and subsequent extraction exactly as in the preparation of actin from muscle pulp[8]. Both of these preparations increased the viscosity of myosin solutions and displayed the G–F transformation and, in the latter case, reacted with actin antibody. It might be argued, however, that the use of organic solvents simply destroys the myosin, producing a fragment that one calls "actin", but which has no significance as far as the original myosin B is concerned. It is presumed that parallel experiments on Myosin A gave no such results, although no mention is made of this point. Furthermore, it has been pointed out that these experiments do not show that the actin is released from combination with myosin *by ATP treatment*[5].

One possible way out of the difficulties outlined above is to investigate the behavior of Myosin B solutions in media in which actin is expected to be entirely in the globular form, *e.g.*, 0.6 M KI or KSCN. Complete depolymerization to monomers should prevent rapid sedimentation of actin and should also wipe out the polydispersity presumably caused by the varying degrees of polymerization of the F-actin molecules. The G-actin peak should then be clearly visible in the ultracentrifuge. With this end in view we have studied the ultracentrifugal, viscometric, and light-scattering properties of solutions of Myosin B in a variety of ionic media.

Incidental to this main objective was the desire to investigate the possible effects of these media themselves on Myosin B, exclusive of their use in identifying the products produced by action of ATP. The early observation that I^- and SCN^- decrease the birefringence of Myosin B solutions[9] and the more recent finding that these ions cause contraction of Myosin B fibers[10] suggest that their action is similar to that of ATP, but the point has never been conclusively demonstrated in solution studies.

Anticipating the results below, we will see that the action of I^- and SCN^- on Myosin B solutions makes it possible to identify G-actin clearly in the ultracentrifuge and to decide the question of ATP action in favor of the classical view of dissociation into myosin and actin moieties. Furthermore, we find, in agreement with expectations, that I^- and SCN^-, and a variety of other ions, themselves dissociate the complex when in proper concentration.

METHODS

Preparations and materials

Myosin B was prepared by 24-h extraction of rabbit muscle with WEBER-EDSALL solution[1], precipitation at ionic strength 0.06 *M*, redissolution in 0.6 *M* KCl, and two more precipitations at 0.28 *M* ionic strength. The final solution (~ 1% protein) was cleaned up by a 3-h centrifugation at 50,000 × *g*.

Myosin was prepared and purified by conventional procedures as previously described[11].

The ATP used was the crystalline disodium salt (Sigma). It was neutralized with potassium hydroxide before use.

All other reagents used were of A.R. grade. Potassium iodide contains enough carbonate impurity so that the pH of fresh solutions is about 9. These were brought to pH 7.2 by adding potassium phosphates to 0.005 *M*. A small amount of sodium thiosulfate (10^{-3} *M*) was added to prevent formation of iodine. Checks of the action of pure iodide, using fresh solutions, showed that the thiosulfate had no detectable effects. Phosphates are similarly inert.

Whenever it was necessary to introduce iodide or thiocyanate ions into the Myosin B solutions by dialysis, this was accomplished in 2 h by stirring thoroughly and making frequent changes of dialyzate. Myosin aggregates rapidly in these media and the solutions must be studied immediately after the addition of these ions.

Sedimentation: All analytical ultracentrifuge runs were made in the Spinco Model E instrument, usually with both the regular and the "wedge window" cell. High speed preparative ultracentrifugations were carried out in the Spinco Model L.

Concentration: Protein concentrations were determined routinely by a Folin procedure, and by microkjeldahl analysis for the physical measurements (*e.g.*, viscosity).

Viscosity: Cannon-Fenske viscometers were used. These required a 5-ml charge and had water efflux times of 200–300 sec.

RESULTS

Action of iodide and thiocyanate ions

As anticipated by various workers[9,10], solutions of Myosin B in 0.6 *M* KI or 0.6 *M* KSCN behave in a manner similar to 0.6 *M* KCl solutions containing ATP.

Fig. 2-a shows the sedimentation pattern displayed by a solution obtained by dialysis *versus* 0.6 M KI. The main peak was found to have $S_{20,w} = 5.9$ S, a value consistent with the identification of this component as myosin. In this medium, actin, if present, would have to be in the globular form. Indeed, in spite of the considerable base line distortion caused by the solvent, a small peak is clearly visible behind the myosin peak, and having $S_{20,w} = 3.5$ S. This value is in satisfactory agreement with that expected from studies on pure actin[12], and is a clear argument in favor of the idea that the actin isolated using the action of organic solvents on muscle pulp (or Myosin B solutions), is not a result of this action, but actually exists as such in the original material. To emphasize this point further we present sedimentation results on a solution in 0.6 M KSCN (Fig. 2-b), which also depolymerizes actin completely, and causes less base line distortion. Again, the results, we feel, are clear cut.

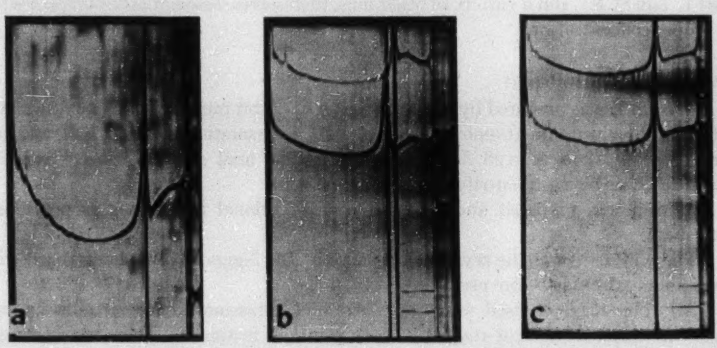

Fig. 2. Centrifugation at 59,780 rev./min; bar angle, 60°. (a) Myosin B in 0.6 M KI, 0.50%; temperature: 12.0°; time, 32 min. For the main peak: $S_{20,w} = 5.9$. For the slow peak: $S_{20,w} = 3.5$. (b) Myosin B in 0.6 M KSCN; temperature, 12.5°; time, 48 min. Wedge cell: 0.80% protein; $S_{20,w} = 4.7$, 3.1. Regular cell: 0.51% protein; $S_{20,w} = 6.0$, 2.9. (c) Myosin in 0.6 M KSCN; temperature, 10.1°; time, 96 min. Wedge and regular cells, 0.88% protein, $S_{20,w} = 3.8$ S.

On the other hand, the addition of iodide or thiocyanate to myosin solutions of higher protein concentration produces no slow peak that could be confused with G-actin (Fig. 2-c). In this case a faint convexity of the base line behind the myosin peak, probably representing traces of actin, is to be compared with the clear peaks seen in Myosin B, at a lower concentration of protein. This is especially noteworthy in view of the recent finding that 0.1 M Na_2CO_3 or 0.01 M KOH do produce a small fragment in both myosin[13] and Myosin B (see ref. 4).

The identification of the slow peak we observed with the actin obtained by acetone treatment of muscle and of Myosin B, or by alcohol treatment of Myosin B solutions, may be made more directly by adding such actin to solutions of Myosin B in 0.6 M KSCN. To this end, actin was prepared from Myosin B by a procedure involving precipitation of the myosin with alcohol[7]. The resulting actin was divided into two aliquots which were dialyzed *vs.* 0.6 M KSCN and 0.1 M KCl + 10^{-3} M $MgCl_2$, respectively. These centrifuge patterns are shown in Fig. 3-a. The thiocyanate solution shows nothing but the expected slow peak. The solution in potassium chloride shows a very small amount of this slow peak and an indeterminate amount of faster material.

Since both solutions contained the same amount of protein it would appear that it is possible to form F-actins that do not show up on the Schlieren plate*.

The actin solution in 0.6 M KSCN, when mixed with Myosin B solution in the same medium, gave the Schlieren pattern shown in Fig. 3-b. The characteristic slow peak of the Myosin B is seen to be augmented by the addition of actin, lending further credence to the view that they are one and the same. It would require an extraordinary coincidence for the action of such different reagents as KI or KSCN, alcohol, and acetone to produce such similar, yet artifactual, substances.

Further proof of the similarity in the effect on Myosin B of KI or KSCN and of ATP was obtained by viscosity measurements (Fig. 4). The intrinsic viscosity of a

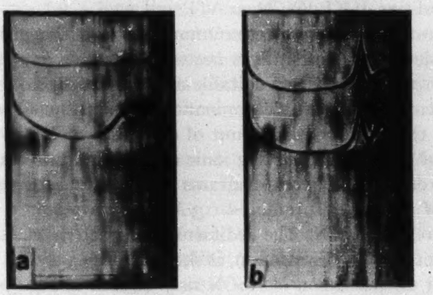

Fig. 3. (a) Actin (prepared from Myosin B) centrifuged at 59,780 rev./min; bar angle, 55°; temperature, 6.8°; time 64 min. Wedge cell: solution in 0.1 M KCl + 10^{-3} M $MgCl_2$. Reg. cell, 0.6 M KSCN; $S_{20,w} = 3.1$ S. (b) Centrifugation at 59,780 rev./min; bar angle 60°; temperature, 14.4°; time, 16 min. Wedge cell: Myosin B, 0.33%, in 0.6 M KSCN; Regular cell: Myosin B, 0.66%, in 0.6 M KSCN + equal volume of actin solution (Fig. 3-A) in 0.6 M KSCN.

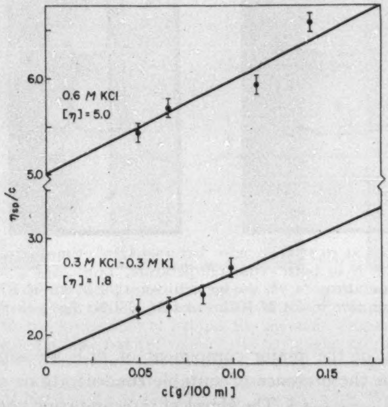

Fig. 4. Intrinsic viscosity of Myosin B in 0.6 M KCl and in 0.3 M KCl + 0.3 M KI.

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* It is worth noting that this F-actin, after dialysis *vs.* 0.6 M KCl, readily formed a very fast moving component when mixed with a myosin solution; and that addition of ATP to this mixture resulted in regeneration of the myosin peak.

Myosin B sample in 0.6 *M* KCl at 25.3 ± 0.02° was found to be 5.0 dl/g. When measured immediately after adding an equal volume of 0.6 *M* KI* to solutions at various protein concentrations, the value fell to 1.8 dl/g. The latter is almost exactly what one would expect for a mixture of myosin and G-actin of the proper composition, and is essentially the value obtained in the presence of ATP[1]. The amount of actin present is sufficiently small so that its presence in the G or F forms makes little difference in the intrinsic viscosity of the solution.

Finally, we have consistently found that light-scattering measurements on these solutions show molecular weights and radii of gyration comparable to those produced by ATP. Qualitatively speaking, there is an immediate and visually obvious reduction in turbidity on addition of KI, KSCN, or ATP.

It is of interest to determine the minimum concentration of I^- or SCN^- required to produce complete dissociation. It is necessary in these experiments to maintain the ionic strength at 0.6 *M*, with a suitable amount of KCl, to retain the protein in solution. For a semiquantitative determination, Myosin B solutions (in 0.6 *M* KCl) were mixed with the appropriate amount of 0.6 *M* KI or KSCN and 0.6 *M* KCl to provide a series of solutions of varying ionic composition but with the same protein concentration. From the Schlieren diagrams it was estimated that at a total ionic strength of 0.6 *M* and 0.5 % protein ~0.3 *M* KI was required for complete dissociation and ~0.2 *M* KSCN. The sedimentation pattern in 0.4 *M* KCl + 0.2 *M* KSCN, indicating complete dissociation, is shown in Fig. 5-a. The partial dissociation caused by a lower concentration of KSCN may be seen in Fig. 5-b.

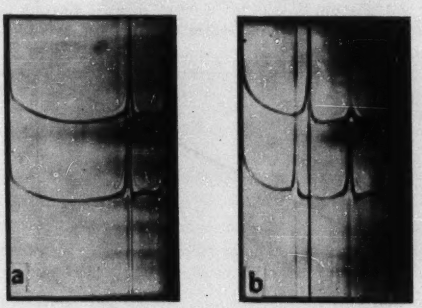

Fig. 5. Myosin B centrifuged at 59,780 rev./min; bar angle, 55°; time, 48 min. (a) 0.34 % protein in 0.4 *M* KCl + 0.2 *M* KSCN in both cells; temperature, 13.0°; $S_{20,w} = 5.2, 3.6$. (b) 0.51 % protein in both cells; temperature, 11.7°. Wedge cell: solution in 0.6 *M* KCl; $S_{20,w} = 11.7, 5.9$. Regular cell: solution in 0.5 *M* KCl + 0.1 *M* KSCN; $S_{20,w} = 13.6, 5.5$ S.

The effects caused by these reagents thus represent definite and positive evidence that a component exists in Myosin B solutions that is necessary for the retention of the molecular integrity of the major component of 24-h Myosin B, and that this component is altered in the presence of suitable concentrations of KI or KSCN to form material with $S_{20,w} \cong 3$–4 S. The simplest interpretation consistent with these observations is that this component is the same as actin prepared from acetone powders of muscle pulp, whose properties it shares, and that, in Myosin B, it is in combination with the other product identifiable after addition of KI or KSCN: myosin. Of course, this does not prove directly that the action of ATP produces actin, but the evidence given here that actin is present in these solutions in combination with myosin and the fact that myosin is produced by ATP leaves little room for doubt. Nevertheless, more evidence on this point will be presented in the next section but one.

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* Thus producing a solution containing 0.3 *M* KCl + 0.3 *M* KI. This is sufficient iodide to cause complete dissociation (see below).

Action of other monovalent anions on Myosin B solutions

The results described above on effects of iodide and thiocyanate prompted us to investigate the effects of other anions on Myosin B.

It has been known for a long time, of course, that high concentrations of KCl cause effects similar to ATP addition[1], but the process has not been studied in detail and no evidence has come forward on whether this is a simple ionic strength effect or a result of chloride binding. These studies, a brief preliminary report of which follows, do not allow for quantitative interpretation, but do suggest that the effects are not simply caused by ionic strength.

Several monovalent anions were tested for their effects on Myosin B solutions at pH 7. In every case the total ionic strength was kept at least at 0.6 *M* by adding enough KCl, where necessary, to make up the difference. In Fig. 6 ultracentrifuge patterns are shown in 0.6 *M* KBr, 0.6 *M* KNO_3, 1 *M* and 2 *M* KCl and in a mixture of composition: 0.3 *M* NaCl + 0.3 *M* $NaClO_4$[*]. Table I indicates the minimum concentration needed to produce complete dissociation in this system for all the anions tested. The order is suggestive of the HOFMEISTER series, but not entirely in register with it. The steady change in minimum concentration of halogenide needed for complete

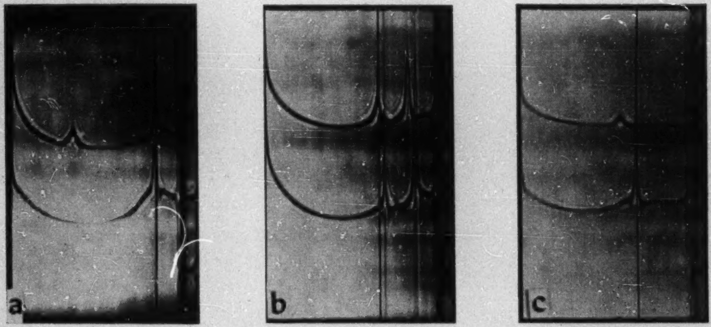

Fig. 6. Myosin B centrifuged in various media at 59,780 rev./min. (a) bar angle, 60°; temperature, 10.6°; time, 32 min. Wedge cell, 0.65 % protein in 0.6 *M* KNO_3; $S_{20,w}$ = 21, 4.7, 4.0 (latter for peak, not yet visible in the frame shown, behind the myosin). Regular cell, 0.65 % protein in 0.6 *M* KBr; $S_{20,w}$ = 4.9, 4.0 (also not yet visible). (b) bar angle, 55°; temperature, 11.0°; time, 32 min. Wedge cell, 0.51 % protein in 1 *M* KCl; $S_{20,w}$ = 11.3, 5.3. Regular cell, 0.51 % protein in 2 *M* KCl; $S_{20,w}$ = 12.9, 4.5. (c) bar angle, 55°; temperature, 12.0°; time, 80 min. Regular cell, 0.51 % protein in 0.3 *M* NaCl + 0.3 *M* $NaClO_4$; $S_{20,w}$ = 4.4, 3.0 S. Wedge cell run is irrelevant in this case.

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[*] Potassium perchlorate is insufficiently soluble so that sodium has to be used as the cation. This has no demonstrable effect, as was shown by comparing solutions in 0.6 *M* NaCl and 0.6 *M* KCl

dissociation makes it doubtful that the dissociation produced by 2 *M* KCl is a simple ionic strength effect.

The question of the reversibility of this phenomenon is not easy to answer, partly because of the fairly rapid aggregation of myosin in these media, and partly because the conversion to G-actin becomes irreversible after a short time. However, preliminary work indicates that at least partial reversibility can be achieved, if the actin is "protected" with ADP[14] and if the exposure time is kept short.

TABLE I

In all cases the total ionic strength was at least 0.6 *M*, the difference being made up for with KCl, where necessary.

Anion	*Minimum concentration for complete dissociation (M)*
Paratoluene sulfonate	0.20
Thiocyanate	0.20
Iodide	0.30
Perchlorate	0.30
Bromide	0.60
Nitrate	0.65
Chloride	$>$ 2.0

Products produced by ATP action

Recent work leaves little room for doubt that myosin is the major product produced by ATP addition to Myosin B solutions[6, 15]. We have already noted that myosin itself shows no $S_{20,w} \cong 3$–4 S peak in 0.6 *M* KSCN. If, then, actin is the other product of dissociation by ATP, the pellet obtained after addition of ATP and centrifugation should show a slow peak in 0.6 *M* KSCN. The result of an experiment in which this pellet was dissolved in a small volume of 0.6 *M* KSCN is shown in Fig. 7. The only visible constituent has $S_{20,w} = 3.4$ S, which enables us to identify the "rapidly sedimenting" product of ATP addition (the "cementing component" of ref. 5), as rich in actin*. Since the "slowly sedimenting" product is myosin this would seem to be conclusive evidence that Myosin B is "actomyosin" and that the addition of ATP produces myosin and actin.

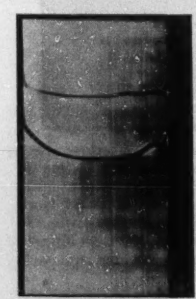

Fig. 7. Pellet produced from Myosin B by ATP addition and centrifugation and dissolved in 0.6 *M* KSCN. Ultracentrifuge run at 59,780 rev./min; bar angle, 60°; temperature, 7.8°; time, 8 min; $S_{20,w} = 3.4$ S in regular cell. Wedge cell run is irrelevant in this case.

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* Of course the pellet should also contain myosin, but since a large portion of the precipitate is insoluble the absence of myosin in the solution obtained is not surprising.

DISCUSSION

There is one further objection that has been raised to the "classical" picture of ATP–Myosin B interaction that is worth mentioning. It has been remarked[5] that dissociation of an actomyosin complex by ATP should lead to "smaller" (*i.e.*, less massive) particles, and that, therefore, it should be easier to destroy the ATP response of Myosin B by centrifugation in the absence of ATP than in its presence—in disagreement with the facts. It would seem reasonable, however, that a thread-like F-actin molecule, relieved of its rod-like myosin partners, would coil up more tightly, thus decreasing its hydrodynamic resistance sufficiently to overcompensate for its diminished mass. Quantitative studies on purified actins are required in order to determine whether this effect is, in fact, occurring.

The work described here concerns only the major components of Myosin B, visible in ordinary Schlieren optics. We can say nothing, therefore, about the large "deformable" component proposed and discussed by GELLERT *et al.*[4]. We wish merely to point out here that Myosin B does contain actomyosin and that one result of ATP action is its dissociation into actin and myosin.

Thus far, only speculation is possible on the mechanism of the dissociation of actomyosin by these anions. The simplest assumption, of course, is that these ions are bound to the individual protein moieties and that charge repulsion leads to dissociation. Such measurements of ion binding by myosin as exist[16] are in rough accord with the data of Table I. Similar data are not available for actin, however, and the fact that the dissociation produced by monovalent ions occurs in the same range of ion concentration as actin depolymerization indicates that the state of charge of the actin is playing as important a role as that of the myosin. Furthermore, since ATP produces F-actin as a dissociation product, the mechanism of action of the nucleotide may differ from that of the other anions, where it is complicated by (or includes as an essential step) G-actin formation.

ACKNOWLEDGEMENT

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REFERENCES

[1] A. SZENT-GYÖRGYI, in *The Chemistry of Muscular Contraction*, Academic Press, New York, N.Y., 1951, 2nd edition.
[2] C. H. ARRINGTON, *Ph. D. Thesis*, California Institute of Technology, 1949.
[3] J. J. BLUM AND M. MORALES, *Arch. Biochem. Biophys.*, 43 (1953) 208.
[4] M. GELLERT, P. H. VON HIPPEL, H. K. SCHACHMAN AND M. MORALES, *J. Am. Chem. Soc.*, 81 (1959) 1384.
[5] P. H. VON HIPPEL, M. GELLERT AND M. MORALES, *J. Am. Chem. Soc.*, 81 (1959) 1393.
[6] A. WEBER, *Biochim. Biophys. Acta*, 19 (1956) 345.
[7] A. G. SZENT-GYÖRGYI, *J. Biol. Chem.*, 192 (1951) 361.
[8] J. GERGELY AND A. MARTONOSI, *Federation Proc.*, 17 (1958) 894.
[9] A. VON MURALT AND J. EDSALL, *J. Biol. Chem.*, 89 (1930) 351.
[10] K. LAKI AND W. BOWEN, *Biochim. Biophys. Acta*, 16 (1955) 301.
[11] A. HOLTZER AND S. LOWEY, *J. Am. Chem. Soc.*, 81 (1959) 1370.
[12] W. F. H. M. MOMMAERTS, *J. Biol. Chem.*, 198 (1952) 445.
[13] D. R. KOMINZ, W. CARROLL, E. SMITH AND E. MITCHELL, *Arch. Biochem. Biophys.*, 79 (1959) 191.
[14] A.G. SZENT-GYÖRGYI, *Arch. Biochem. Biophys.*, 31 (1951) 97.
[15] P. H. VON HIPPEL, H. SCHACHMAN, P. APPEL AND M. MORALES, *Biochim. Biophys. Acta*, 28 (1958) 504.
[16] B. N. GHOSH AND E. MIHALYI, *Arch. Biochim. Biophys.*, 41 (1952) 107.

THE BINDING OF CYANOCOBALAMIN AND ITS NATURALLY OCCURRING ANALOGUES BY CERTAIN BODY FLUIDS AND TISSUE EXTRACTS

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SUMMARY

Chicken serum, an extract of chicken proventriculi and crude and purified extracts of pig pyloric mucosa combined with about equal amounts of cyanocobalamin, factor A, pseudovitamin B_{12} or factor B. In contrast, normal human gastric juice bound factor B significantly less. Preferential binding of cyanocobalamin from mixtures with its naturally occurring analogues was demonstrated with human gastric juice and preparations from pig stomach mucosa. The mechanism whereby animals absorb and retain cyanocobalamin in their body tissues in preference to the other naturally occurring analogues is discussed.

INTRODUCTION

The naturally occurring analogues of vitamin B_{12} are, with the exception of vitamin B_{12III}, inactive in curing pernicious anaemia in man, and are unable to replace vitamin B_{12} as animal growth factors[1]. These analogues are known to be present in appreciable quantities in fermented materials and to arise in the rumen or intestine by microbial synthesis.

It is well established that in man the absorption of vitamin B_{12} depends on the presence of an "intrinsic factor" in the gastric juice and there is evidence that combination with specific glycoproteins plays an important part in the absorption and retention of vitamin B_{12} in man and other animals[2]. We have already shown that the vitamin B_{12} analogues—factor A, pseudovitamin B_{12} and factor B—are bound to the same extent as cyanocobalamin** by concentrates prepared from sow's milk and from desiccated pig stomach[3]. Since body tissues contain predominantly vitamin B_{12} with only a trace of the other factors present, it is evident that animals either have some mechanism for preferentially absorbing vitamin B_{12} from mixtures with its analogues, or some mechanism whereby vitamin B_{12} and its analogues are all absorbed from the intestine but only vitamin B_{12} is retained[4,5].

In this paper some experiments are described which show that the binding

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[**] The term cyanocobalamin denotes a particular compound whereas vitamin B_{12} refers to the naturally occurring vitamin, which is not necessarily in the cyano-form.

components from pig stomach and human gastric juice combine preferentially with cyanocobalamin in the presence of its naturally occurring analogues. This work was briefly referred to by KON[5]. We have also extended our previous studies on the binding of factor A, pseudovitamin B_{12} and factor B by concentrates from sow's milk and pig stomach[3] to include human serum and gastric juice, chicken serum and a chicken proventriculus extract (*i.e.* an extract prepared from the glandular stomach of the chicken).

MATERIALS

A bulk sample of human serum from pernicious anaemia patients, and a sample of neutralized normal human gastric juice were kindly given to us by Dr. D. L. MOLLIN (Postgraduate Medical School, London). The chicken serum was obtained by exsanguination of freshly killed animals. The chicken proventriculus extract was prepared as described by GREGORY AND HOLDSWORTH[2]. Bendogen is a commercial preparation of "intrinsic factor" from pig pyloric mucosa (G.E.A., Copenhagen). Two purified intrinsic factor concentrates were prepared from it. Preparation 1 was obtained by fractionation on a DEAE cellulose ion exchange column as described by HOLDSWORTH[6] and preparation 2 by treatment with acid and papain as described below. Extomak is a commercial brand of desiccated hog stomach prepared by Benger's Ltd., Holmes Chapel, Cheshire (Great Britain). The concentrate was prepared from it as described elsewhere[3]. [^{60}Co]cyanocobalamin of specific activity 42 μC/mg, and [^{60}Co]factor B of specific activity, 200 μC/mg and unlabelled cyanocobalamin (Cytamen) were kindly supplied by Glaxo Laboratories Ltd., Greenford, Middlesex (Great Britain). Factor A, pseudovitamin B_{12} and factor B were purified, and standard solutions prepared as described by GREGORY AND HOLDSWORTH[3]. [^{60}Co]-pseudovitamin B_{12} was prepared by the method of PFIFFNER, DION AND CALKINS[7]. Vitamin B_{12III} was kindly given by Professor K. BERNHAUER, Aschaffenburger Zellstoffwerke, A.G., Stockstadt-am-Main (Germany).

Purified intrinsic factor

Preparation 2. 20 g of Bendogen were suspended in 200 ml of 10 % (v/v)HCl. The insoluble material was removed by centrifuging and the clear supernatant liquid immediately dialysed to remove the HCl. The dialysed solution was incubated at pH 4.5 for 6 h at 40° with the addition of 100 mg of papain (British Drug Houses Ltd) activated with cyanide. The digest was then dialysed against running water for 48 h and then against several changes of distilled water before freeze-drying. The 2.5 g of dry material obtained bound 1.23 μg of cyanocobalamin/mg as measured by the ultrafiltration method[8]. Dr. D. L. MOLLIN (Post graduate Medical School, London) very kindly tested the preparation in a patient with pernicious anaemia and found that it still had intrinsic factor activity.

METHODS

Binding of the analogues of vitamin B_{12}

The binding of cyanocobalamin, factor A, pseudovitamin B_{12} and factor B by the various test materials was measured by the ultrafiltration method of GREGORY

AND HOLDSWORTH[8]. The ultrafiltrates containing the unbound cyanocobalamin, factor A or pseudovitamin B_{12} were assayed with both *Escherichia coli* mutant 113-3 and *Lactobacillus leichmannii* ATCC 4797 as the test organisms[9]. Since factor B is inactive for *L. leichmannii*, the ultrafiltrates containing it were assayed only with *E. coli*. The analogues of vitamin B_{12} each differ in their growth promoting activity for these two test microorganisms and therefore separate standards containing known amounts of the analogues were included in each test. The amounts of the analogues bound by the test materials was calculated in the same way as for cyanocobalamin[8], but by reference to the standard of the particular analogue under test.

With certain of the test materials the binding activities were measured with [^{60}Co]cyanocobalamin, [^{60}Co]pseudovitamin B_{12} and [^{60}Co]factor B as described by GREGORY AND HOLDSWORTH[10].

Preferential binding of cyanocobalamin in the presence of its analogues

Measurement by a bioautograph technique: An aqueous solution of the concentrate from Extomak, sufficient to bind 1.0 μg of cyanocobalamin, was added to mixtures containing 0.8, 0.7, 0.6, 0.5 or 0.4 μg each of cyanocobalamin, factor A, pseudovitamin B_{12} and factor B. The resulting solutions, at final volumes of 10 ml in 0.9 % (w/v) saline, were ultrafiltered. Any unbound factors passed into the ultrafiltrates. Their presence was then detected by taking 10 μl of each ultrafiltrate and subjecting it to ionophoresis on paper in 1 *N* acetic acid[11]. By the bioautograph technique of FORD AND HOLDSWORTH[12], with *E. coli* as the test organism and 2,3,5-triphenyltetrazolium chloride included in the agar medium, the positions of the separated factors were revealed as red zones of growth on a colourless background. In addition to acting as a visual aid in detecting the areas of growth on the plate, the red formazan pigment, formed by reduction of the 2,3,5-triphenyltetrazolium chloride, can be used for the quantitative estimation of the amount of factor present on the plate. The pigment from each zone was eluted with tetrahydrofuran and the amount of colour compared with that obtained on the same plate with spots containing known amounts of the different factors.

Measurement with [^{60}*Co*]*cyanocobalamin*: An aqueous solution of the purified papain-treated Bendogen, sufficient to bind about 1.3 μg of cyanocobalamin, was mixed with a solution containing 4 μg of [^{60}Co]cyanobocalamin either alone or with 4 or 20 μg of unlabelled factor A, pseudovitamin B_{12}, factor B, or vitamin B_{12III}. The volumes were made to 10 ml in 0.9 % saline and the solutions were ultrafiltered. Free cyanocobalamin was determined from the amount of radioactivity present in the ultrafiltrate[10].

RESULTS

Binding of the analogues of vitamin B_{12}

The amounts of cyanocobalamin, factor A, pseudovitamin B_{12} and factor B bound by normal human gastric juice, human serum and chicken serum and by a chicken proventriculus extract and crude and purified extracts from pig pyloric mucosa are shown in Table I. The figures in this table, for results of microbiological tests used to measure the unlabelled analogues, are mean values from at least three assays. However, the amounts of radioactive compounds available at the time were sufficient for duplicate estimations in only one test with each binding factor.

TABLE I

MEAN VALUES FOR THE AMOUNTS OF CYANOCOBALAMIN, FACTOR A, PSEUDOVITAMIN B_{12} AND FACTOR B BOUND BY CERTAIN BODY FLUIDS AND TISSUE EXTRACTS

		Activity in the ultrafiltrate measured by:	*Cyanocobalamin bound µg/ml*	*Factor A bound µg/ml*	*Pseudovitamin B_{12} bound µg/ml*	*Factor B bound µg/ml*
Human gastric juice (normal)		*E. coli*	0.14	0.12	0.08	0.04
		L. leichmannii	0.15	0.12	0.11	—
		Radioactivity	0.12	—	—	—
Human serum (Pernicious anaemia)	Sample 1	*E. coli*	0.0031	0.0046	0.0080	approx. 0.012
		L. leichmannii	0.0018	0.0033	0.0039	—
	Sample 2	Radioactivity	0.0020	—	—	0.0009
Chicken serum	Sample 1	*E. coli*	0.50	0.54	0.62	0.70
		L. leichmannii	0.62	0.67	0.62	—
	Sample 2	*L. leichmannii*	1.10	1.01	1.01	—
		Radioactivity	1.10	—	—	1.00
			µg/g	*µg/g*	*µg/g*	*µg/g*
Chicken proventriculus extract		*E. coli*	27	33	36	33
		L. leichmannii	33	41	37	—
		Radioactivity	33	—	—	24
Pig pyloric mucosa extract (Bendogen)		*E. coli*	210	220	240	210
		L. leichmannii	204	220	220	—
		Radioactivity	200	—	240	260
Purified intrinsic factor	Preparation 1	*L. leichmannii*	9,600	10,000	9,500	—
		Radioactivity	10,000	—	—	11,800
	Preparation 2	*E. coli*	1,230	—	—	—
		Radioactivity	1,300	—	1,400	—

The two samples of chicken serum, the proventriculus extract, and the crude and purified pig pyloric mucosa extracts combined with about equal amounts of cyanocobalamin and its analogues when these were tested individually. This finding confirms our previous work with sow's milk and a pig stomach extract, when a different technique for measuring binding activity was used[3].

The human gastric juice and serum showed different patterns of behaviour. The gastric juice bound slightly less factor A and pseudovitamin B_{12} than cyanocobalamin. The binding of factor B was significantly less than for cyanocobalamin. The opposite effect was observed with the human serum, which appeared to combine with more factor B than cyanocobalamin. However, it was observed that all the values for binding activity obtained when *E. coli* was used to test the vitamin activity of the serum ultrafiltrates were higher than when *L. leichmannii* was the test organism. *L. leichmannii* requires lower concentrations of cyanocobalamin, factor A and pseudovitamin B_{12} for growth, and therefore the ultrafiltrates can be tested at a more dilute concentration. We have often noticed that *E. coli* does not grow as well with factor B as with cyanocobalamin. When known amounts of factor B were added to an ultrafiltrate of human serum, 70 % of it was available to *E. coli* and when cyanocobalamin was added to the ultrafiltrate, a full recovery was obtained with both *L. leichmannii* and *E. coli*. Thus, serum may contain factors which slightly inhibit the growth of *E. coli* in the presence of factor B, but not in the presence of cyanocobalamin. However, an inhibition of some 30 % of the growth activity of factor B does not entirely account for the very low activities measured in these ultrafiltrates. The human serum and gastric juice were both tested at the same time as the Bendogen, so that it was certain that the different results were not due to some error in the technique of measurement of the binding activity. The one test done with [^{60}Co]-factor B on a different sample of serum indicated that less factor B was bound than cyanocobalamin.

Preferential binding of cyanocobalamin in a mixture of analogues

When a mixture containing 0.8 μg each of cyanocobalamin, factor A, pseudo vitamin B_{12} and factor B was added to a pig stomach preparation, which could bind only 1.0 μg of vitamin B_{12}-like factors, about equal amounts of each factor appeared in the ultrafiltrate. This finding is shown in Table II and indicates that under these

TABLE II

PREFERENTIAL BINDING BY A PIG STOMACH PREPARATION OF CYANOCOBALAMIN IN THE PRESENCE OF EQUAL AMOUNTS OF FACTOR A, PSEUDOVITAMIN B_{12} AND FACTOR B

10 mg of Extomak concentrate bind 1 μg of vitamin B_{12}-like factors.

Amount of each factor added (μg)	*Amount of each factor bound expressed as a percentage of the amount added*			
	Cyanocobalamin	*Factor A*	*Pseudovitamin B_{12}*	*Factor B*
0.8	31	29	31	26
0.7	34	28	31	27
0.6	38	28	33	27
0.5	53	38	46	37
0.4	93	58	69	48

conditions no one of the factors was preferentially bound. As the proportion of cyanocobalamin and its analogues to binding factor was reduced, so the amount of cyanocobalamin bound increased. Finally when 0.4 μg of each factor were present, almost all the cyanocobalamin had become bound, while substantial amounts of the other factors still appeared in the ultrafiltrate. Similar results were obtained with normal human gastric juice, which was tested with 0.8 μg or 0.4 μg of each factor. All factors appeared in the ultrafiltrate at the higher level of addition, but the cyanocobalamin did not at the lower level of addition.

In a further series of experiments the combination of [^{60}Co]cyanocobalamin with the purified pig pyloric mucosa extract was measured in the presence of different compounds structurally related to cyanocobalamin. The results are given in Table III. They show that when equal amounts of cyanocobalamin and of one other structurally related compound competed for a limited amount of binding factor, cyanocobalamin was preferentially bound. Even in the presence of a five-fold excess of the competing compound, substantial amounts of cyanocobalamin were still bound.

TABLE III

COMPETITION BETWEEN [^{60}Co]CYANOCOBALAMIN AND ITS ANALOGUES FOR THE BINDING SITES IN PURIFIED PAPAIN-TREATED BENDOGEN

Analogue added	*μg [^{60}Co]cyanocobalamin bound/mg of Bendogen*	
	4 μg analogue present	*20 μg analogue present*
None	1.3	1.3
Factor A	0.9	0.8
Pseudovitamin B_{12}	0.9	0.5
Factor B	0.8	0.7
Vitamin B_{12III}	0.8	0.5

DISCUSSION

The *in vitro* experiments, described in this paper, have shown that there is a marked difference in the behaviour of the binding factors of human origin and those from the pig and chicken. When three of the naturally occurring analogues of cyanocobalamin —factor A, pseudovitamin B_{12} and factor B—were tested individually with chicken serum or extracts of chicken proventriculi or of pig pyloric mucosa, each of them was bound to the same extent as cyanocobalamin. The human serum also bound each of the analogues, but possibly more so than cyanocobalamin. In contrast to these findings, the human gastric juice bound much less factor B and probably less factor A and pseudovitamin B_{12} than cyanocobalamin.

We have previously shown that cells of the protozoan *Ochromonas malhamensis*, grown with limiting cyanocobalamin, were able to take up about equal amounts of factor A, pseudovitamin B_{12} or cyanocobalamin when these were added individually to the medium[13]. Very little, if any, factor B was taken up. We have since found that extracts of disrupted *Ochromonas* cells can combine with cyanocobalamin, factor A and pseudovitamin B_{12} and render them non-ultrafiltrable. Factor B was not bound.

Thus the uptake of the analogues of cyanocobalamin by *Ochromonas* cells and cell extracts resembles the binding of these analogues by human gastric juice.

Our results with normal human gastric juice point to a different type of binding mechanism from the one present in human serum and in extracts of pig pyloric mucosa. BUNGE, SCHLOESSER AND SCHILLING[14] came to a similar conclusion for human gastric juice and serum. In *in vitro* experiments they showed that pseudovitamin B_{12} did not interfere with the binding of [^{60}Co]cyanocobalamin by gastric juice, whereas it competed with [^{60}Co]cyanocobalamin for the binding sites in human serum. Furthermore, an oral dose of 50 μg of pseudovitamin B_{12} had no effect on the absorption of 1 μg [^{60}Co]cyanocobalamin administered with 50 ml of normal human gastric juice to a pernicious anaemia patient. COATES, DAVIES, DAWSON, HARRISON, HOLDSWORTH, KON AND PORTER[15] have shown that for the chick, large amounts of pseudovitamin B_{12}, given orally, antagonized the growth promoting effect of cyanocobalamin, although if both were given by injection there was no adverse effect. They concluded that its antagonistic effect was due to competition with cyanocobalamin for the absorption mechanism. If one can compare chick growth tests with clinical tests, it would seem that the mechanism of absorption of vitamin B_{12} differs in the chicken and in man, the one being antagonized by pseudovitamin B_{12}, whereas the other is not. An antagonistic effect of pseudovitamin B_{12} on the growth of *Ochromonas* has been reported by FORD[16]. In this respect therefore the absorption mechanism in *Ochromonas* resembles that in the chicken. However, the two differ in that factor A is an inhibitor and factor B inactive for *Ochromonas* whereas factor A is inactive and factor B an inhibitor of growth for the chick[15].

Our *in vitro* experiments also show that under certain conditions cyanocobalamin can be preferentially bound in the presence of its analogues. One of the binding factors used for these tests was a crude preparation from desiccated pig stomach (Extomak), which is a potent source of both "intrinsic factor" and cyanocobalamin-binding activity. Cyanocobalamin-binding activity is not synonymous with "intrinsic factor" activity, and indeed HOLDSWORTH[6] has shown that the pig pyloric mucosa preparation (Bendogen) can be purified by fractionation on a DEAE cellulose column and two similar substances with binding activity obtained—only one of which has "intrinsic factor" activity. We show here that such a purified "intrinsic factor" preparation from Bendogen bound equal amounts of each of the analogues when they were tested individually. Unfortunately this preparation was not tested for preferential binding of cyanocobalamin in the presence of its analogues, but a sample of normal human gastric juice with intrinsic factor activity showed the same preferential uptake of cyanocobalamin as the crude pig stomach preparation.

Our *in vitro* experiments were limited by the fact that we were able to study only those tissue extracts or body fluids that have high binding activities. However, our results do show that the absence of factor A and pseudovitamin B_{12} from body tissues is not due to their inability to enter into combination with the binding factors in the intestinal tract. It is more likely that these binding factors, or the receptor sites on the intestinal wall, or the body tissues themselves, have a higher affinity for cyanocobalamin than for its analogues and that the analogues, if absorbed, are quickly excreted. The high proportion of the factor A, pseudovitamin B_{12} and factor B in cow's urine[5] supports this theory.

REFERENCES

[1] M. E. COATES AND S. K. KON, *Vitamin B_{12} und Intrinsic Factor*, Hamburg, 1956, p. 72.
[2] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 72 (1959) 549.
[3] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 55 (1953) 830.
[4] J. E. FORD, E. S. HOLDSWORTH AND J. W. G. PORTER, *Proc. Nutrition Soc. (Engl. and Scot.)*, 12 (1953) xi.
[5] S. K. KON, *Biochem. Soc. Symposia (Cambridge, Engl.)*, 13 (1955) 17.
[6] E. S. HOLDSWORTH, *Biochem. J.*, 66 (1957) 59P.
[7] J. J. PFIFFNER, H. W. DION AND D. G. CALKINS, *Federation Proc.*, 11 (1952) 269.
[8] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 59 (1955) 329.
[9] M. E. GREGORY, *Brit. J. Nutrition*, 8 (1954) 340.
[10] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 66 (1957) 456.
[11] E. S. HOLDSWORTH, *Nature*, 171 (1953) 148.
[12] J. E. FORD AND E. S. HOLDSWORTH, *Biochem. J.*, 53 (1953) xxii.
[13] J. E. FORD, M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 61 (1955) xxiii.
[14] M. B. BUNGE, L. L. SCHLOESSER AND R. F. SCHILLING, *J. Lab. Clin. Med.*, 48 (1956) 735.
[15] M. E. COATES, M. K. DAVIES, R. DAWSON, G. F. HARRISON, E. S. HOLDSWORTH, S. K. KON AND J. W. G. PORTER, *Biochem. J.*, 64 (1956) 682.
[16] J. E. FORD, *J. Gen. Microbiol.*, 19 (1958) 161.

METAL BINDING PROPERTIES OF CHONDROITIN SULPHATE

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SUMMARY

The interactions of H^+, Ca^{2+}, Y^{3+}, La^{3+}, with chondroitin sulphate of cartilage have been studied using a micropotentiometric titration technique. These ions are bound to the carboxyl groups of chondroitin sulphate by the formation of 1:1 complexes, with intrinsic association constants in KCl solution (with the ionic strength in parentheses); H^+ $\log K = 3.06 \pm 0.02$ (0.1); 2.92 ± 0.02 (1.0); Ca^{2+} $\log K = 0.42 \pm 0.09$ (1.0); Y^{3+} $\log K = 0.61 \pm 0.05$ (1.0); La^{3+} $\log K = 0.79 \pm 0.03$ (1.0). The electrostatic contribution to the binding constant, due to the charge of the polymeric molecule, accords with the "nearest-neighbour interaction" description of the titration curve. The influence of the sulphate groups on these results is discussed and is considered small.

INTRODUCTION

Chondroitin sulphate is a poly-β-D-glucopyranosyluronic acid 2-deoxy-2-acetylamino-galactose sulphate[1], which occurs in bone, and as a major constituent of cartilage, probably in the form of chondromucoprotein, in which it is bound to a noncollagenous protein[2]. There appear to be two closely related forms, the A and C isomers, in which the ester sulphate group is in position 4 and 6 of the galactose ring respectively[3].

The fundamental importance of this polyelectrolyte in providing sites for the deposition of calcium ions during the calcification of cartilage has often been postulated[4], and it has also been suggested as binding radio–yttrium in the mammalian skeleton[5]. In spite of these hypotheses no precise measurements have hitherto been reported of the interactions of these metal ions with purified chondroitin sulphate. In this paper is reported a potentiometric titration study of these systems, and also of the interactions with hydrogen and with lanthanum ions.

EXPERIMENTAL

2-ml aliquots of solutions containing up to 10 mg of polysaccharide were titrated with magnetic stirring in small flat-bottomed glass tubes, using standard size wide-range glass electrodes, with agar bridge connections to a saturated calomel electrode. Titrant was added from a calibrated "Agla" microburette with a well-fitting glass capillary nozzle. The ionic strength of the solutions was either 0.1 *M* or 1.0 *M*, allowing the use of high concentrations of di- or trivalent metal ion in the presence of excess KCl. Owing to the difficulty of handling small volumes of solution in carbon dioxide-free conditions, the whole titration assembly was enclosed in a specially constructed glove box of low leakage rate, containing CO_2-free nitrogen at a small positive pressure. For ease of use several titration vessels were mounted on a rotating stand. A Cambridge Bench Type pH meter was used outside the glove box, connection being made with the electrodes through screened bulkhead fittings. Temperatures were 22 ± 2° throughout.

Stock calcium solutions were made from analytical grade $CaCl_26H_2O$ followed by determination of Ca^{2+} with oxalate; yttrium solutions from 99% pure Y_2O_3 and lanthanum solutions from "Specpure" La_2O_3 (both obtained from Messrs. Johnson, Matthey and Co. Ltd.), by dissolution in HCl and removal of excess acid. Two sources of mucopolysaccharide were used: (a) chondroitin sulphate supplied by Messrs. L. Light and Co. Ltd., prepared from alkaline extracts of bovine trachea and thus a mixture of the A and C isomers[6]; (b) chondromucoprotein obtained by aqueous extraction of bullock nasal septum, by the method of MALAWISTA AND SCHUBERT[2]. Material (a) was subsequently further purified by a cetyltrimethyl ammonium bromide precipitation procedure[7], and the final product contained N, 2.27%; S, (see ref. 8) 5.10%; glucuronic acid (by titration), 31.8%. Theoretical values for $C_{14}H_{19}NSO_{14}K_2 \cdot 4\frac{1}{2}H_2O$ are N, 2.27%; S, 5.16%; glucuronic acid 31.4%. This preparation migrated on paper electrophoresis as a single band, staining metachromatically with toluidine blue; two small u.v. fluorescent bands shown by the crude material were removed by the purification, which also lowered the N content by about 0.4%. The spectrophotometric absorption at 280 mμ, partially attributed to protein contaminants, of a 2 mg/ml solution in 1-cm cells was lowered by the purification to 0.080, which accords with that of material after repeated precipitation on kaolin[9].

RESULTS

Hydrogen ion binding

The degree of H^+ association has been derived from the titration curve of the mucopolysaccharide by comparison with the corresponding titration curve in the absence of mucopolysaccharide. A small correction has been made at the lower pHs for volume differences between the two solutions. The results are plotted in Fig. 1. The chondroitin sulphate titration has two distinct regions: on the acid side the carboxyl groups are titrated and on the alkaline side about 2.5 % of a carboxyl equivalent is titrated with a pK of about 8.2. The possibility of titration of the ester sulphate groups in the more acid solutions was investigated by a graphical method, which also gave an accurate equivalent weight.

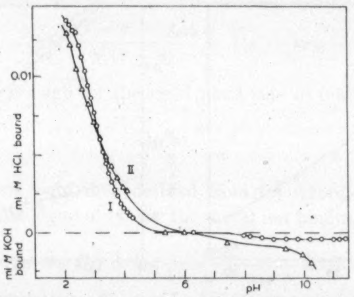

Fig. 1. H$^+$ binding of chondroitin sulphate (curve I), and of a chondromucoprotein preparation (curve II).

At the ionic strengths used the comparatively wide spacing of the carboxyl groups along the chondroitin sulphate chain permits analysis of the titration curve in terms of the "nearest neighbour interaction" model of Hill[10]. The unit of the linear polymer may be regarded for this purpose as the trisaccharide in which a glucuronic acid ring is flanked by two sulphated acetylamino-galactopyranose rings, and the relevant equation becomes:

$$\log K = \mathrm{pH} + \log \frac{\alpha}{1-\alpha} - A(1-\alpha) \tag{1}$$

where α = the degree of association of protons with the carboxyl groups, K = the intrinsic association constant, A = the electrostatic coefficient.

If y = the bound protons in moles/l, a = the concentration of chondroitin sulphate in carboxyl equivalents/l and h = the activity of hydrogen ions, incorporating the mean activity coefficient, (1) may be written

$$\log K - \mathrm{pH} = \log \frac{y}{a-y} - A(1 - y/a) \tag{2}$$

hence

$$y = a(1 - y/Kah.\ \varepsilon^{-2.30A(1-y/a)}) \tag{3}$$

For y/a near unity, that is at low pH, since A is small, (3) approximates to:

$$y = a(1 - By/h) \tag{4}$$

where B is an almost constant factor.

A plot of y against y/h (Fig. 2) should be linear with $y = a$ as its extrapolated intercept, and hence the equivalent weight may be calculated. Binding of protons to the sulphate groups at lower pHs would cause enhanced values of y and positive deviations from linearity. The plots of Fig. 2 therefore show that such binding is negligible within the experimental range, that is down to pH 2.

Equation (2) shows that, for this model, pH + log $y/(a-y)$ should be linear with $y/a = \alpha$, with an intercept at $\alpha = 1$ of log K, and a slope of $-A$. This is demonstrated in Fig. 3, and the derived constants are given in Table I.

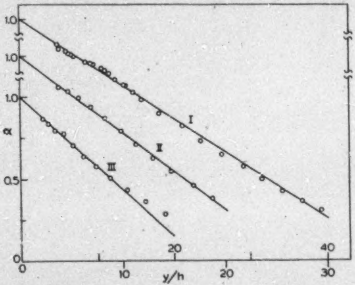

Fig. 2. Determination of equivalent weight by extrapolation (curves I and II in 1 M solution, lower scale; curve III in 0.1 M solution, upper scale).

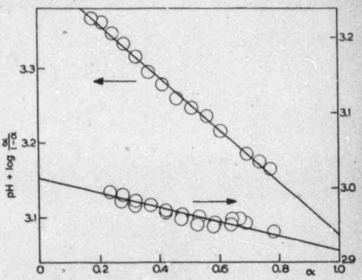

Fig. 3. The electrostatic contribution to the association constant, and the determination of the intrinsic constant.

On the acid side the titration curve of the chondromucoprotein preparation is broadly similar to that of chondroitin sulphate, as suggested by its composition[2]. The differences in the alkaline titrations are discussed below.

TABLE I

INTRINSIC ASSOCIATION CONSTANTS

Cation	*Ionic strength*	*A*	*log₁₀ K*
H^+	0.10	0.354	3.06 ± 0.02
H^+	1.00	0.098	2.92 ± 0.02
Ca^{2+}	1.00	0.098	0.42 ± 0.09
Y^{3+}	1.00	0.098	0.61 ± 0.05
La^{3+}	1.00	0.098	0.79 ± 0.03

Calcium binding

In the presence of excess calcium, lanthanum, or yttrium ions, the acid titration curve of chondroitin sulphate was displaced to more acid values, indicating association of metal ions with the carboxyl groups. The displacements were small, and measurements were restricted to the higher ionic strength to permit the use of high concentrations of added metal ion. The degree of proton binding was derived as described above, using as blank a titration curve of the same salt solution. The low hydrolysis of Ca^{2+} allowed alkaline titrations also, and here no displacement of the titration curve could be detected, indicating negligible complex formation with the corresponding groups.

In the presence of metal ion the analogue of equation (1) is:

$$\log K = \text{pH} + \log \frac{\alpha'}{1 - \alpha' - \alpha_M} - A(1 - \alpha' - n\alpha_M) \qquad (5)$$

where α', α_M, are the degrees of association of H^+ and of M^{n+} respectively at the carboxyl groups. Considering pairs of titrations, identical except for the presence or absence of M^{n+}, at the same pH, (1) and (5) give:

$$\log \frac{\alpha'}{1 - \alpha' - \alpha_M} - A(1 - \alpha' - n\alpha_M) = \log \frac{\alpha}{1 - \alpha} - A(1 - \alpha) \tag{6}$$

$$\therefore \log \frac{\alpha(1 - \alpha' - \alpha_M)}{\alpha'\,(1 - \alpha)} = A(\alpha' + n\alpha_M - \alpha) \tag{7}$$

For Ca^{2+} α_M is small enough for the right hand side to tend to zero, and

$$\alpha_M = \frac{\alpha - \alpha'}{\alpha} \tag{8}$$

Better approximations were then derived from (7) where necessary by successive approximation. The analogue of (5) for the metal ion binding is:

$$\log K_M = -\log C_M + \log \frac{\alpha_M}{1 - \alpha' - \alpha_M} - nA(1 - \alpha' - n\alpha_M) \tag{9}$$

where C_M = the concentration of metal ion, corrected for the small proportion bound to the polysaccharide, and K_M = the intrinsic association constant, in terms of ionic concentrations at constant ionic strength. Hence using αM derived from (8) and (7), $\log K_M$ follows from (9). These results are tabulated in Table II, and the mean

TABLE II

TITRATION RESULTS

Cation	*pH*	α'	α_M	$nA(1\text{-}\alpha'\text{-}n\alpha_M)$	$\log_{10} K_M$
La^{3+} (0.05 M)	3.64	0.130	0.207	0.07	0.74
	3.48	0.174	0.219	0.05	0.82
	3.16	0.302	0.188	0.04	0.84
	3.08	0.345	0.169	0.04	0.80
	2.94	0.423	0.142	0.04	0.78
	2.83	0.492	0.123	0.04	0.77
	2.63	0.599	0.102	0.03	0.81
	2.46	0.691	0.074	0.03	0.78
Y^{3+} (0.1 M)	3.59	0.135	0.256	0.03	0.61
	3.44	0.174	0.253	0.03	0.60
	3.32	0.216	0.242	0.02	0.64
	3.22	0.258	0.243	0.00	0.69
	3.14	0.300	0.207	0.02	0.61
	3.05	0.337	0.208	0.01	0.66
	2.92	0.415	0.174	0.02	0.61
	2.81	0.489	0.139	0.03	0.55
	2.61	0.605	0.101	0.03	0.51
Ca^{2+} (0.1 M)	3.42	0.274	0.133	0.09	0.26
	3.30	0.327	0.153	0.07	0.40
	3.21	0.368	0.124	0.07	0.31
	3.13	0.414	0.134	0.06	0.41
	2.99	0.493	0.115	0.05	0.41
	2.86	0.562	0.098	0.05	0.41
	2.64	0.665	0.084	0.03	0.49
	2.47	0.746	0.073	0.02	0.59
	2.33	0.795	0.052	0.02	0.51

log K_{Ca} in Table I. In the derivation of all the log K_M, values have been taken within a range of 0.5 pH units on either side of the mid-point of the titration curve where percentagewise errors are minimal.

Lanthanum and yttrium binding

These metals are more strongly complexed than calcium and the results have been calculated by an alternative method. At any point on the titration curve all quantities of (5) are known, using the value of K already derived, except α_M, which can be calculated by successive approximations. In the first step the electrostatic contribution is put equal to zero, and in following steps is included using the approximated α_M. A maximum of four steps was found adequate. K_M was then calculated at each point by (9). Results are given in Tables I and II.

DISCUSSION

The most accurate of previous determinations of the pK of the carboxyl groups of chondroitin sulphate are those of MATHEWS[11] who gives intrinsic association constants at 0.1 M ionic strength only: log $K = 3.07 \pm 0.08$ and 3.06 ± 0.06 for two different preparations from bovine nasal septum. The agreement with corresponding results of the present work is within the quoted experimental error. Comparison with earlier determinations[12] is uncertain, because of invalid derivations of the intrinsic constants.

The alkaline titration curve shows the presence of about 2 groups, which are titrated completely between pH 7 and 9, with log $K = 8.2$, for each 100 carboxyl groups present. No further titration is apparent, at least up to pH 11.6. This behaviour is quite unlike that of mucoprotein preparations (Fig. 1) and, together with the analyses, suggests that the groups are not those of protein contaminants. This accords with the conclusion of LOEVEN[12], and the groups in question are considered to be primary amino groups formed by slight hydrolysis of acetylamino groups during extraction. The negligible Ca^{2+} complexing with these groups supports this identification.

The metal ion binding data have been evaluated assuming a "single site" association between metal ions and carboxyl groups, which is confirmed by the lack of drift in K_M values with increasing carboxyl ionisation. In any case the single site association is weak, and steric factors hinder linkage of a metal ion to groups on more than one ring (see ref. 13). The association constants are in fact very close to those for isolated carboxyl groups; they may be compared with published values for acetate ions: Ca^{2+} in 0.2 M ionic strength, log $K = 0.53$ (see ref. 14), La^{3+} in 2.0 M ionic strength, log $K = 0.49$ (see ref. 15), although differences in ionic strength do not allow exact comparison. The comparatively small Ca^{2+} association explains why an earlier qualitative study of the system failed to demonstrate complex formation[16].

The solutions used have contained high concentrations of chloride ion and allowance for metal-chloride association should be made in applying the results to different systems. For Ca^{2+} and Y^{3+} the effects seem slight or absent[17,18], but for La^{3+} the $La^{3+}/LaCl^{2+}$ ratio at the same ionic strength[19] necessitates an increase of 0.20 in log K_{La} in chloride free systems.

A possible cause of small systematic errors in this work is ion-pair formation between metal ions and the ester sulphate groups. Firstly, this could affect the calculated concentration of free metal ion; however, the greatest error possible is about the same as the experimental error in the case of La^{3+}, and considerably less for Ca^{2+} and Y^{3+}. Secondly, this could introduce an additional electrostatic contribution to the binding constant. It is considered that binding of these metal ions to the sulphate groups in the present experimental conditions of moderate to high ionic strength is small. This is shown by the high concentration (0.33 *M*) of La^{3+} needed to reverse the mobility of adsorbed chondroitin sulphate in microelectrophoretic measurements[20]. In controlled conditions such mobilities are closely related to the charge on the adsorbed polyelectrolyte[21], and at this concentration the present results show that sufficient La^{3+} ions are bound to the carboxyl groups to neutralise the whole charge on the molecule, and it is unnecessary to postulate appreciable pairing with the sulphate groups. Similar considerations apply to Ca^{2+}, and to Y^{3+} by implication. Thus, although small degrees of association at the sulphate groups are not impossible their influence on the present results is slight.

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REFERENCES

[1] E. A. DAVIDSON AND K. MEYER, *J. Am. Chem. Soc.*, 77 (1955) 4796.
[2] I. MALAWISTA AND M. SCHUBERT, *J. Biol. Chem.*, 230 (1958) 535.
[3] A. G. LLOYD AND K. S. DODGSON, *Nature*, 184 (1959) 548.
[4] J. T. IRVING, *Calcium Metabolism*, Methuen & Co. Ltd., London, 1957, p. 128.
[5] J. M. VAUGHAN, in G. H. BOURNE, *Bone*, Academic Press Inc., New York, 1956, p. 740.
[6] K. MEYER, E. DAVIDSON, A. LINKER AND P. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506.
[7] M. STACEY, A. B. FOSTER AND B. C. BERA, *J. Chem. Soc.*, (1955) 3788.
[8] K. S. DODGSON AND B. SPENCER, *Biochem. J.*, 55 (1953) 436.
[9] J. EINBINDER AND M. SCHUBERT, *J. Biol. Chem.*, 185 (1950) 725.
[10] T. L. HILL, *J. Am. Chem. Soc.*, 78 (1956) 5527.
[11] M. B. MATHEWS, *Biochim. Biophys. Acta*, 35 (1959) 9.
[12] W. A. LOEVEN, *Koninkl. Ned. Akad. Wetenschap. Proc. Ser. B*, 60 (1957) 23.
[13] C. ROBINSON, *Discussions Faraday Soc.*, 16 (1954) 125.
[14] R. K. CANNAN AND A. KIBRICK, *J. Am. Chem. Soc.*, 60 (1938) 2314.
[15] A. SONESSON, *Acta Chem. Scand.*, 12 (1958) 165.
[16] J. R. DUNSTONE, *Biochem. J.*, 72 (1959) 465.
[17] E. C. RIGHELLATO AND C. W. DAVIES, *Trans. Faraday Soc.*, 26 (1930) 592.
[18] K. A. KRAUS, F. NELSON AND G. W. SMITH, *J. Phys. Chem.*, 58 (1954) 11.
[19] R. E. CONNICK AND W. H. MCVEY, *J. Am. Chem. Soc.*, 75 (1953) 474.
[20] P. H. TEUNISSEN AND H. G. BUNGENBERG DE JONG, *Kolloid-Beih.*, 48 (1939) 80.
[21] I. G. F. GILBERT, *Biochim. Biophys. Acta*, 40 (1960) 156.

FRACTIONATION OF HYALURONIC ACID

THE POLYDISPERSITY OF HYALURONIC ACID FROM THE BOVINE VITREOUS BODY*

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SUMMARY

The polydispersity of hyaluronic acid from the bovine vitreous body has been demonstrated by the successful separation of the polysaccharide into fractions with molecular weights ranging from $7.7 \cdot 10^4$ to $1.7 \cdot 10^6$. The results of light-scattering, ultracentrifuge and viscosity studies have been compared; it has been found that, where discrepancies occur, the limiting viscosity numbers correspond more closely to the molecular weights obtained by light-scattering than to those evaluated from ultracentrifuge data. The extent to which light-scattering and ultracentrifuge molecular weights are at variance appears to be a function of the degree of polydispersity of each individual sample.

The relationship between the limiting viscosity number and molecular weight, $[\eta] = 0.036 \cdot M^{0.78}$ is expected for a coil configuration for hyaluronic acid.

INTRODUCTION

While there is general agreement that hyaluronic acid from the bovine vitreous body has a lower molecular weight than that from most other sources, the molecular weight as reported in the literature varies between $5.7 \cdot 10^4$ and $1.3 \cdot 10^6$ (see ref.1–9). There is usually a large discrepancy between values obtained by sedimentation and diffusion[4,8,9] and light-scattering[2,3,6–8], the former being lower than the latter. The rather large variability of data has been ascribed, among other things, to methods of preparation, to the use of several physicochemical methods in the determination of molecular weight, and finally, and perhaps most importantly, to the effects of polydispersity of the polymer. Viscosity studies on ammonium sulfate fractionated material[10] and on material isolated from different parts of the bovine vitreous body[11], as well as light-scattering[3], diffusion[12] and streaming birefringence studies[1,6], and sedimentation boundary analysis[4] on unfractionated material, have indeed indicated that hyaluronic acid from the bovine vitreous body is highly polydisperse.

This paper describes the fractionation of hyaluronic acid, obtained in high yield, into fractions of different molecular weight. The fractions obtained have been studied by light-scattering, sedimentation and diffusion analysis, and viscosity.

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* Paper 87, Retina Foundation.

METHODS

Refractive increment

The refractive increment of sodium hyaluronate was determined in a calibrated Rayleigh-type Zeiss interferometer at a wavelength of 546 mμ at 25°. Measurements were made at several concentrations on unfractionated sodium hyaluronate that had been carefully dialyzed against 0.1 *M* sodium chloride. The concentrations of the samples on which readings were taken were determined on a dry-weight basis. Equal volumes of dialysate and sodium hyaluronate solutions were dried over sulfuric acid at 60° for 48 h and the dry weight was obtained as the weight difference.

Light-scattering

Measurements were made at room temperature in a BRICE-SPEISER instrument[13] (Pheonix Precision Instrument Co.) at a wavelength of 436 mμ, and the angular distribution of scattered light was measured at angles between 35° and 135° with respect to the incident beam. The specimens were centrifuged in calibrated glass cells similar to those described by DANDLIKER AND KRAUT[14] for 2 h at 24,800 × *g*. In order to minimize the effects of the concentration gradients obtained during centrifugation, the cells were allowed to stand in the cold for 24 h before readings were taken. Blank readings were obtained from centrifuged sodium chloride solutions. The concentration range employed in each series of experiments was dictated by the molecular weight of the particular fraction under study.

Sedimentation

Determination of sedimentation velocities using the moving boundary method were made at 52,640 rev./min at room temperature in a Spinco Model E analytical ultracentrifuge equipped with a Philpot-Svensson cylindrical lens optical system. Sedimentation coefficients (S_{20}) were obtained at five concentrations for each fraction. Extrapolation to infinite dilution was obtained by plotting $1/S$ against concentration[15].

Diffusion

Diffusion experiments were conducted in the analytical ultracentrifuge with valve-type synthetic-boundary cells. Solvent was layered over sodium hyaluronate solutions at speeds of 7 to 10,000 rev./min, and the speed was then reduced to 5,000 rev./min. Diffusion coefficients (D_{20}) were calculated by the height–area method[16] from photographic enlargements using a planimeter for area measurements. 6 to 8 determinations sufficed to permit a linear extrapolation to zero concentration.

Viscosity

Limiting viscosity numbers were calculated for each fraction at 25°. The measurements were made over a concentration range of 0.15 to $5.6 \cdot 10^{-3}$ g/ml, using Ostwald viscometers with outflow times of 40 to 50 sec/ml and shear gradients of the order of 1,000 cm^{-1}.

Chemical methods

Hexuronic acid was measured by the carbazole method[17], using glucuronic acid as a standard, and sodium hyaluronate concentrations were then calculated from hexuronic acid values by multiplication with a factor of 1.9 found empirically from dry-weight determinations. A modification of the Elson-Morgan method[18,19] was used to determine hexosamine. Total nitrogen values were obtained by a micro Kjeldahl analysis[20] and the protein content was calculated from non-hyaluronic acid nitrogen (protein = N × 6.25).

Preparation and fractionation techniques

Preparation. The occipital part of the vitreous body collected from fresh steer and cow eyes as described earlier[3] was liquified by centrifugation for 2 h at 65,000 × g and dialyzed against several changes of distilled water at 4°. Hyaluronic acid was isolated as cetylpyridinium hyaluronate by the addition of 0.11 g of cetylpyridinium chloride (Kebo Co., Stockholm) per 100 ml of liquid vitreous[21]. The cetylpyridinium hyaluronate precipitate was collected by centrifugation and suspended in 2 M sodium chloride. Constant stirring for 2 days at room temperature dissolved all but a small part of the precipitate. The insoluble matter, although finely dispersed, resisted all efforts to bring it into solution. Micro-cel E (Johns Manville Co.) was now added at the rate of 3.5 g/100 ml to remove both cetylpyridinium chloride and the remaining protein impurities[22]. After having been stirred for 2 h, the suspension was centrifuged for 20 min at 35,000 × g and the supernatant was collected. To minimize the loss of hyaluronic acid it was necessary to resuspend the Micro-cel E in an equal volume of 2 M sodium chloride, centrifuge again, and collect the supernatant. The supernatants were combined and the solution was analyzed.

At this stage the sodium hyaluronate was dialyzed against 0.2 M sodium chloride and precipitated from solution by the addition of 3 volumes of ethanol. The material was stored in the cold in the aqueous ethanol. The sodium hyaluronate was then redissolved in concentrations suitable for the work to be performed.

Fractionation. A solution of 1.77 g of cetylpyridinium chloride in 50 ml of 0.25 N Na_2SO_4 was added to 1.24 g of sodium hyaluronate dissolved in 205 ml of the same medium. The slightly turbid solution was centrifuged for 15 min at 75,000 × g and the small amount of precipitate obtained was labeled fraction I. The supernatant was then diluted with distilled water in successive stages to Na_2SO_4 concentrations of 0.190, 0.174, 0.165, 0.155, 0.130 and 0.060 N and after each dilution the solution was centrifuged. The sediments collected constituted fractions II through VII. Each precipitate was dissolved in 2.5 N Na_2SO_4 and treated with Micro-cel E to remove cetylpyridinium chloride. This was followed by dialysis against 0.2 M sodium chloride, chemical analysis and reprecipitation with 3 volumes of ethanol.

The solution remaining after separation of fraction VII was treated with Micro-cel E. Subsequent addition of 3 volumes of ethanol precipitated a large amount of salt as well as sodium hyaluronate (fraction VIII). This precipitate was then dissolved in distilled water and dialyzed at + 4°. A precipitate formed on addition of 3 volumes of ethanol to the dialyzed solution was collected as fraction VIII A. Storage of the turbid supernatant in the cold and the addition of a small amount of sodium chloride gave a second precipitate (fraction VIII B).

All physicochemical studies were made on sodium hyaluronate dialyzed against

0.2 *M* sodium chloride unless otherwise stated. Fractions VI and VII were combined before the physicochemical studies.

RESULTS

Fractionation

Sodium hyaluronate was isolated from bovine vitreous body in 89 % yield with a protein contamination of less than 2 %. Table I summarizes the results of the preparative procedure. The data tabulated represent the combined material obtained from three separate preparations.

TABLE I

THE ISOLATION OF SODIUM HYALURONATE

	Original vitreous humor	*Preparation*	*Recovery*
Sodium hyaluronate	1.359 g	1.210 g	89.2 %
Protein	1.415 g	0.0185 g	1.3 %
Ratio sodium hyaluronate: protein	0.96:1	65.5:1	

Table II outlines the results obtained in the fractionation procedure. The recovery was 79 %, which gave an overall yield of 70 % for preparation and fractionation. There seems to be no indication of any difference in the chemical composition of the various fractions. The discrepancy often encountered between the methods we used for hexosamine and hexuronic acid analysis[17, 23] was observed.

TABLE II

ANALYTICAL DATA

	Normality of Na_2SO_4 at precipitation	*Size of fraction mg*	*Percent of starting material*	*Hexosamine: hexuronic acid: nitrogen molar ratio*
Unfractionated sodium hyaluronate, sample A*	0.00			0.85:0.94:1.00
Unfractionated sodium hyaluronate, sample B*	0.00			0.90:1.05:1.00
Fraction I	0.250	—	—	
II	0.190	11	0.9	
III	0.174	58	4.7	0.88:0.96:1.00
IV	0.165	135	10.9	0.85:0.93:1.00
V	0.155	260	20.9	0.81:0.99:1.00
VI	0.130	152	12.3	0.82:0.94:1.00
VII	0.060	28	2.2	
VIIIA	—	144	11.6	0.81:0.99:1.00
VIIIB	—	192	15.5	0.88:0.96:1.00
Recovery		980 mg	79.0 %	

* Samples A and B and a third preparation were pooled for the fractionation experiment.

Refractive increment

The refractive increment of sodium hyaluronate was found to be 0.166 at 546 mμ. Since this is substantially in agreement with the refractive increments reported earlier[24], there was no need to recalculate the constant K of the light-scattering equation.

Light-scattering

The data were treated and evaluated according to ZIMM[25]. $K \cdot c/R_\theta$ was plotted against $\sin^2 \theta/2 + kc$, where K is a constant, c the concentration, R_θ the reduced angular intensity of the scattering, θ the observation angle and k an arbitrary constant. The value $4.58 \cdot 10^{-7}$ was used for the constant K (see ref. 24). A double extrapolation to zero angle and zero concentration gave the reciprocal molecular weight. The radius of gyration was calculated from the slope of the initial angular scattering envelope and its intercept on the $K \cdot c/R_\theta$ axis. Representative ZIMM-plots are given in Figs. 1 and 2.

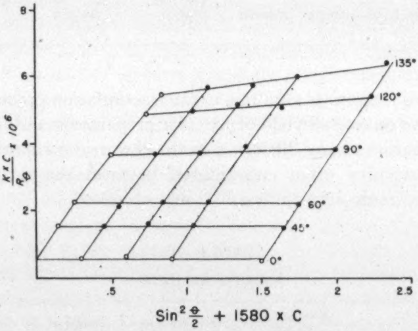


Fig. 1. ZIMM-plot of fraction III. Mol. wt. $1.7 \cdot 10^6$. Radius of gyration 1430 Å.

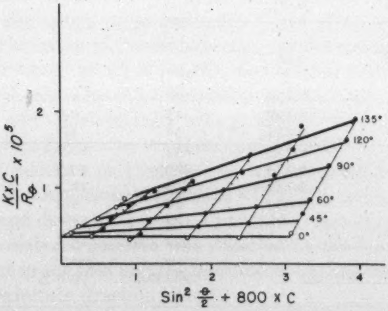


Fig. 2. ZIMM-plot of fraction VI–VII. Mol. wt. $2.8 \cdot 10^5$. Radius of gyration 540 Å.

The values for molecular weight and radius of gyration obtained from the light-scattering data are tabulated in Table III. The errors of the determinations are estimated to be of the order of ± 10 %[24]. In the case of the fractions of lowest molecular weight the data were not reliable enough to warrant calculation of the radius of gyration.

TABLE III

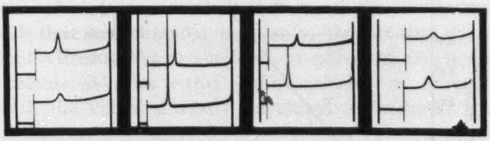
PHYSICOCHEMICAL DATA

Fractions	*Ultracentrifuge data*			*Light-scattering data*		Limiting viscosity number ml/g
	Sedimentation constant $S_{20,w} \times 10^{13}$	*Diffusion constant* $D_{20,w} \times 10^{7}$	*Molecular weight*	*Molecular weight*	*Radius of gyration Å*	
Unfractionated sodium hyaluronate, sample A	2.98	0.99	$2.2 \cdot 10^5$	$3.7 \cdot 10^5$	640	945
Unfractionated sodium hyaluronate, sample B	3.13	0.80	$2.8 \cdot 10^5$	$4.5 \cdot 10^5$	710	970
Fraction III	7.15	0.34	$1.5 \cdot 10^6$	$1.7 \cdot 10^6$	1430	2450
IV	6.67	0.38	$1.3 \cdot 10^6$	$1.3 \cdot 10^6$	1570	2340
V	3.76	0.65	$4.1 \cdot 10^5$	$3.8 \cdot 10^5$	630	875
VI–VII	3.12	1.16	$1.9 \cdot 10^5$	$2.8 \cdot 10^5$	540	700
VIIIA	2.52	1.89	$9.5 \cdot 10^4$	$1.1 \cdot 10^5$	—	295
VIIIB	2.46	1.74	$1.0 \cdot 10^5$	$7.7 \cdot 10^4$	—	250

Sedimentation

Fig. 3 depicts the sedimentation patterns of unfractionated hyaluronic acid, and of samples of high, medium and low molecular weight. It can be seen that the sharpness of the boundary varies directly with molecular weight and concentration.

Fig. 4 shows the concentration dependence of the reciprocal sedimentation coefficient. The sedimentation velocity is independent of molecular weight at concentrations above $2 \cdot 10^{-3}$ g/ml. The sedimentation constants are listed in Table III.



Fraction	*Unfractionated NaHyaluronate B*	*III*	*V*	*VIII-A*
Concn., (mg/ml)	1.40 0.90	0.88 1.33	0.70 1.41	0.96 1.44
Time (min)	140	141	144	152
Bar angle	60°	60°	60°	55°

Fig. 3. Sedimentation patterns.

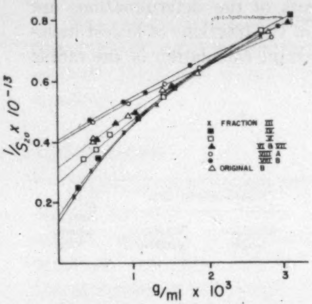


Fig. 4. Reciprocal sedimentation velocity as a function of concentration.

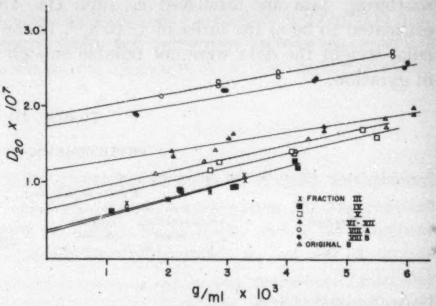
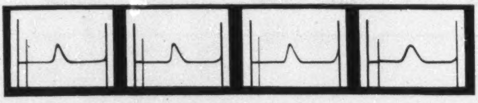


Fig. 5. The diffusion coefficient as a function of concentration.

Diffusion

Diffusion has been studied as a function of concentration (Fig. 5). A linear relationship was observed in each case, and the concentration dependence was found to increase with the molecular weight. The marked differences in concentration dependence of the diffusion characteristics of the individual fractions were manifested



Fraction	*Unfractionated NaHyaluronate B*	*III*	*V*	*VIII–A*
Concn., (mg/ml)	2.71	3.30	2.80	2.87
Time (min)	154	159	153	152
Bar angle	60°	65°	60°	55°

Fig. 6. Diffusion patterns.

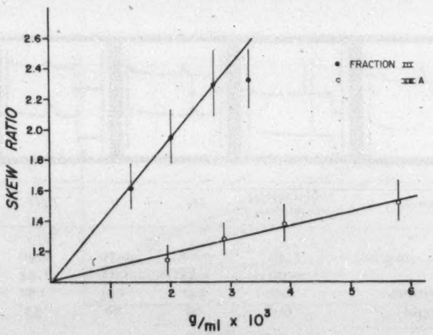


Fig. 7. The skew ratio (see text) of diffusion patterns as a function of concentration. The vertical lines represent the effects of a theoretical 2 % error in the area determinations.

as a variation in the skewness of the corresponding diffusion patterns (Fig. 6). Gosting[16] has reviewed this interrelationship from a theoretical standpoint. The ratio of the areas on either side of the maximum was used as a measure of skewness (Fig. 7). In accordance with the theoretical expectations, extrapolation of the skew ratios to infinite dilution gave a value of one.

Molecular weights were calculated from sedimentation and diffusion data according to the Svedberg formula with use of the value 0.66 cm^3/g for the partial specific volume of hyaluronic acid[4]. Owing to the difficulties in the extrapolation procedures, the molecular weight values (Table III) have errors of the same magnitude as those obtained from light-scattering measurements.

Viscosity

The viscosity data plotted as a function of concentration are shown in Fig. 8 and the extrapolated limiting viscosity numbers are given in Table III.

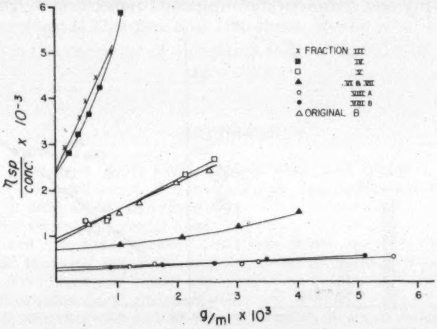


Fig. 8. Viscosity data.

DISCUSSION

The conclusions that can be deduced from the data gathered in this investigation are of particular interest if the hyaluronic acid studied is representative of that existing in the tissue from which it was isolated. That this is the case is supported not only by the high yields that were obtained, but also by the fact that the molecular weight of unfractionated vitreous hyaluronic acid prepared by cetylpyridinium chloride precipitation—calculated from either light-scattering or sedimentation-diffusion data—agrees with the values similarly calculated for material prepared by other methods[3,4,7–9].

The results show the molecular weight of unfractionated hyaluronic acid to be $4 \cdot 10^5$, when evaluated from light-scattering measurements, and $2.5 \cdot 10^5$, when determined from sedimentation and diffusion data. This discrepancy, which has also been reported in earlier work, might be explained by the demonstrated polydispersity of bovine vitreous hyaluronic acid[8] (Fig. 9). Moore and Greear[26], in recent studies on fractionated polyethylenes, have found that large molecules, which exert a great influence on light-scattering measurements, in low concentration escape detection in the ultracentrifuge.

It is apparent then that the weight-average molecular weights of polydisperse materials tend to be underestimated by ultracentrifugal methods, whereas light-scattering methods yield values that are more correct. The agreement between the two methods improves the more homogeneous is the system. The calculated weight-average molecular weight of unfractionated hyaluronic acid obtained from the molecular weights of the fractions is $4.5 \cdot 10^5$ and is in close agreement with the light-scattering value. Similarly, the limiting viscosity number of unfractionated hyaluronic acid corresponds to a molecular weight which is closer to that found by light-scattering than to that obtained from ultracentrifuge results.

The data seem to indicate that there may be two species of hyaluronic acid in the vitreous, one with a molecular weight of the order of $1.5 \cdot 10^6$, the other with a molecular weight below $4 \cdot 10^5$ (Fig. 9). These have been isolated as fractions III and IV, and fractions V–VIII, respectively. With the exception of fractions VI–VII, the fractions were judged to be relatively homogeneous on the basis of the agreement between light-scattering and sedimentation-diffusion molecular weights.

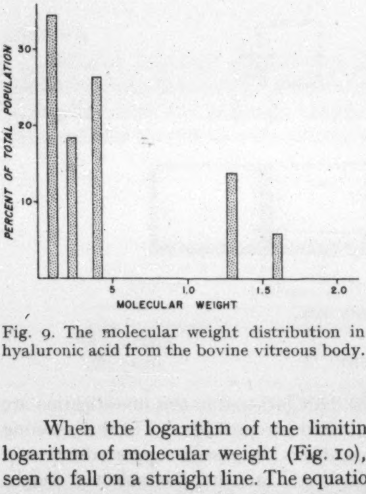


Fig. 9. The molecular weight distribution in hyaluronic acid from the bovine vitreous body.

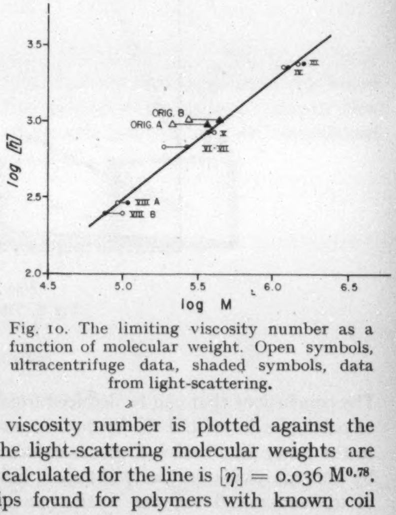


Fig. 10. The limiting viscosity number as a function of molecular weight. Open symbols, ultracentrifuge data, shaded symbols, data from light-scattering.

When the logarithm of the limiting viscosity number is plotted against the logarithm of molecular weight (Fig. 10), the light-scattering molecular weights are seen to fall on a straight line. The equation calculated for the line is $[\eta] = 0.036\ M^{0.78}$. The equation agrees with the relationships found for polymers with known coil configurations[27]. Similarly, the proportionality observed between the radius of gyration and the limiting viscosity number ($(\bar{r}^2)^{1/2} = k[\eta]^{0.85}$), as well as the functional relationship observed between radius of gyration and molecular weight ($(\bar{r}^2)^{1/2} = k \cdot M^{0.60}$), is close to that expected for a coil.

The concentration dependence of sedimentation velocity is greatest at low concentrations and this complicates the evaluation of sedimentation constants. The hazards of extrapolating the data to infinite dilution have been minimized by plotting the reciprocal sedimentation velocity as a function of concentration (Fig. 4). At high concentrations the sedimentation velocity is independent of molecular weight, indicating that no longer are single molecules sedimenting, but rather a continuous three-dimensional network[28].

The concentration dependence of the diffusion coefficients imparts a definite skewness to the diffusion patterns (Fig. 6). Under these circumstances, erroneous values are obtained when diffusion coefficients are calculated by standard methods. However, since the skewness disappears at infinite dilution (Fig. 7), diffusion constants obtained by extrapolation of data to zero concentration should be correct.

The wide variance in the values reported for the refractive increment of hyaluronic acid[6,24,29,30] prompted a reinvestigation. In order to avoid the errors inherent in chemical analysis, the concentration of the samples on which readings were taken were determined directly as dry-weights. It is felt that this procedure and the purity of the preparation enhance the reliability of our data. Actually, a value in agreement with our earlier results[24] has been obtained.

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REFERENCES

[1] G. Blix and O. Snellman, *Arkiv Kemi, Mineral. Geol.*, 19A (1945) 1.
[2] R. Brunish, J. W. Rowen and S. R. Irvine, *Trans. Am. Ophthalmol. Soc.*, 52 (1954) 369.
[3] T. C. Laurent, *J. Biol. Chem.*, 216 (1955) 263.
[4] L. Varga, *J. Biol. Chem.*, 217 (1955) 651.
[5] J. A. Christiansen and E. C. Jensen, *Acta Chem. Scand.*, 9 (1955) 1405.
[6] J. W. Rowen, R. Brunish and F. W. Bishop, *Biochim. Biophys. Acta*, 19 (1956) 480.
[7] T. C. Laurent, *Arkiv Kemi*, 11 (1957) 487.
[8] E. A. Balazs, *Federation Proc.*, 17 (1958) 1086.
[9] L. Varga, A. Pietruszkiewicz and M. Ryan, *Biochim. Biophys. Acta*, 32 (1959) 155.
[10] K. Meyer, *Physiol. Rev.*, 27 (1947) 335.
[11] E. A. Balazs and L. Sundblad, personal communication.
[12] R. Brunish, M. Vangerov and W. Irvine, *Federation Proc.*, 15 (1956) 226.
[13] B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Am.*, 40 (1950) 768.
[14] W. B. Dandliker and J. Kraut, *J. Am. Chem. Soc.*, 78 (1956) 2380.
[15] H. K. Schachman, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959.
[16] L. J. Gosting, *Advances in Protein Chem.*, 11 (1956) 429.
[17] Z. Dische, *J. Biol. Chem.*, 167 (1947) 189.
[18] L. A. Elson and W. T. J. Morgan, *Biochem. J.*, 27 (1933) 1824.
[19] E. A. Balazs, personal communication.
[20] T. S. Ma and G. Zuazaga, *Ind. Eng. Chem., Anal. Ed.*, 14 (1942) 280.
[21] J. E. Scott, *Biochim. Biophys. Acta*, 18 (1955) 428.
[22] E. A. Balazs and L. Sundblad, *Acta Soc. Med. Uppsaliensis*, 64 (1959) 137.
[23] S. Gardell, *Methods of Biochemical Analysis*, 6 (1958) 289.
[24] T. C. Laurent and J. Gergely, *J. Biol. Chem.*, 212 (1955) 325.
[25] B. H. Zimm, *J. Chem. Phys.*, 16 (1948) 1099.
[26] L. D. Moore, Jr. and G. R. Greear, *136th Meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1959.*
[27] P. J. Flory and T. G. Fox, Jr., *J. Am. Chem. Soc.*, 73 (1951) 1904.
[28] A. G. Ogston and E. F. Woods, *Trans. Faraday Soc.*, 50 (1954) 635.
[29] B. S. Blumberg and G. Oster, *Science*, 120 (1954) 432.
[30] B. S. Blumberg, A. G. Ogston, D. A. Lowther and H. J. Rogers, *Biochem. J.*, 70 (1958) 1.

PHOSPHORYLASE AND URIDINEDIPHOSPHOGLUCOSE-GLYCOGEN TRANSFERASE IN PYRIDOXINE DEFICIENCY

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SUMMARY

The total phosphorylase activity of the skeletal muscle of rats maintained on a pyridoxine deficient diet has been found to fall to 35 % of the normal value. The phosphorylase *a* activity of the tissue of these rats has the normal value, and the glycogen content of the muscles of such deficient rats is not different from that of control animals. The apparent activity of uridine diphosphoglucose–glycogen transferase is not changed from the normal level in pyridoxine deficiency.

INTRODUCTION

It has been shown that phosphorylase *a* contains 4 moles of pyridoxal-5′-phosphate per mole of enzyme, and that the apoenzyme prepared by ammonium sulfate precipitation from acid solution is inactive in the absence of added pyridoxal-5′-phosphate[1,2]. Since other enzymes requiring pyridoxal-5′-phosphate have been shown to decline in activity in Vitamin B_6 deficient animals[3], it was of interest to investigate the activity of phosphorylase in the muscle of rats made deficient in B_6. The activity of UDPG-glycogen transferase was also measured, since this enzyme has been shown to be active *in vivo* in lengthening a preexisting glycogen chain[4,5], and, thus, to have an action like that of phosphorylase.

MATERIALS, METHODS AND RESULTS

In the first series of experiments weanling albino rats (Sprague-Dawley) were divided into two groups. The control group was fed laboratory chow, and the experimental group was given a pyridoxine-deficient diet supplied by Nutritional Biochemicals Corporation. After 7 to 8 weeks the animals were anesthetized and muscle samples were removed as follows. Both gastrocnemii were exposed *in situ* and then cut out in rapid succession. A 100–200 mg sample of each was taken to determine its glycogen content in duplicate. The remaining tissue was weighed and homogenized for 3 min in a Virtis homogenizer with 9 volumes of ice-cold 0.05 *M* Tris–0.001 *M* EDTA,

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine-tetraacetic acid; UDPG, uridine diphosphoglucose; UDP, uridine diphosphate.

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pH 7.5. For phosphorylase determinations an aliquot of this 1:10 homogenate was immediately diluted five-fold in ice-cold 0.001 *M* EDTA–0.02 *M* NaF, pH 6.0. The fluid medium used for homogenization was chosen so that phosphorylase and UDPG–glycogen transferase could both be determined[6]. For the analysis of the latter enzyme, an aliquot of the uncentrifuged homogenate was incubated for 15 min at 30° in the presence of $1.5 \cdot 10^{-3}$ *M* UDPG, 1.0% of added glycogen, $7.5 \cdot 10^{-3}$ *M* cysteine, $1.2 \cdot 10^{-2}$ *M* $MgCl_2$, $1.0 \cdot 10^{-3}$ *M* EDTA, $1 \cdot 10^{-1}$ *M* Tris, pH 7.5. The reaction was stopped by heating the mixture for 2 min in boiling water, followed by cooling in ice, centrifugation, and determination of the amount of UDP formed *via* the pyruvate kinase–lactic dehydrogenase system[7].

The immediate dilution of the homogenate with EDTA–NaF served to stop subsequent action of the specific phosphatase (PR-enzyme) and specific kinase known to change the relative proportion of phosphorylases *a* and *b*. Once diluted in this manner, the ratio of *a* to *b* remained constant during a period of several days. The phosphorylase *a* activity was determined at 30° at pH 6.0 in the presence of 0.016 *M* glucose-1-phosphate and 1% of added glycogen. Total phosphorylase activity was determined as above, with the addition of $1 \cdot 10^{-3}$ *M* 5′-adenylic acid (5′-AMP).

The data in Table I show that the only significant change exhibited by the deficient animals was a decrease in the total phosphorylase activity to 35% of that found in the control rats. Incubation of the homogenate under conditions which lead to the reactivation of the apoenzyme of phosphorylase[8], did not result in any increase of total activity. When the original 1:10 homogenates of the muscles from deficient animals were stored overnight at 5° or incubated at 30° for short periods of time, a decrease in phosphorylase activity in the absence of added 5′-adenylic acid was observed, showing that the PR-enzyme (acting to change $a \rightarrow b$) was active. When the fresh homogenates from the deficient animals were supplemented with ATP and

TABLE I

EFFECT OF PYRIDOXINE DEFICIENCY ON ENZYME ACTIVITY AND GLYCOGEN CONTENT

Animal	*Glycogen content (% wet wt.)*	*Phosphorylase activity (units*/g tissue)*		*UDPG-glycogen transferase activity (units**/g tissue)*
		— 5′-AMP	*+ 5′-AMP*	
Control group				
1	0.56	1060	9990	17.0
2	0.73	487	8910	22.9
3	0.57	165	7890	17.9
4	0.50	1290	8940	20.0
Deficient group				
5	0.58	822	4185	20.2
6	0.44	876	3195	17.0
7	0.66	540	2580	18.8
8	0.50	1040	3510	12.0
9	0.54	675	2525	17.6
10	—	595	2400	19.7

* For detailed description of assay see ref. 2.

** One unit of activity is the number of µmoles of UDPG which are converted to UDP under the assay conditions described in the text.

Mg^{++}, there occurred an increase in phosphorylase *a* activity, indicating that phosphorylase *b* kinase was active.

In a second series of experiments the weanling rats were divided into two groups. One group was fed the pyridoxine-deficient diet used above. The second group was fed the "Vitamin B Complex Test Diet (with complete Vitamin Fortification)", supplied by Nutritional Biochemicals Corporation. Thus, each group of rats received food of similar physical appearance. The first rats of each group were killed after two weeks, at which time the level of phosphorylase in the deficient rats was already depressed. For these measurements and for all subsequent ones, the initial homogenization of the excised muscle was done in EDTA–NaF.

At 3.5 weeks, four rats were changed from the B_6 deficient diet to the complete diet. These rats were sacrificed at intervals following resumption of B_6 intake. As shown by the data in Table II, these rats showed an increasing level of total phosphorylase activity which approached normal values after 4 weeks of access to the complete diet.

TABLE II

TIME COURSE OF PHOSPHORYLASE ACTIVITY CHANGE IN PYRIDOXINE DEFICIENCY

Animal	*Rat weight (g)*	*Days on diet*	*Phosphorylase activity (units*/g tissue)*	
			— 5′-AMP	*+ 5′-AMP*
Control group				
11	86	15	928	7320
12	130	24	460	8200
13	164	35	820	9260
14	178	45	—	8520
15	166	51	1160	9440
16	170	51	1140	9240
Deficient group				
17	54	15	828	3750
18	66	15	595	3360
19	70	24	1135	3350
20	72	24	860	3140
21	72	35	1020	2980
22	90	45	930	2190
23	89	51	720	2300
Deficient group after supplementation**				
24	110	11**	960	6000
25	152	21	700	6000
26	146	21	660	4600
27	148	27	930	9930

* For detailed description of assay see ref. 2.

** On deficient diet for 24 days and then on complete diet (see text) for time shown.

DISCUSSION

The data in Table II show that the level of phosphorylase *a* in pyridoxine-deficient rats remains relatively constant while that of total phosphorylase ($a + b$) shows a marked drop. To some extent this shifting of the *a* to *b* ratio in the muscle appears to be a mechanism for maintaining a nearly normal potentiality for glycogen metabolism in the B_6 deficient animal. The extent of decline in the total phosphorylase level is about that which has been seen in other enzymes having pyridoxal-5′-phosphate as a prosthetic group[3].

The UDPG–glycogen transferase activity of the deficient animals was not significantly different from that of the control group. This finding does not suggest any role of pyridoxal-5′-phosphate in the reaction catalyzed by this enzyme. It should be pointed out, however, that if only the data for phosphorylase *a* activity and not those for total phosphorylase activity as well, were available from the present experiments, a similar conclusion would seem justified. Of course, in the light of other facts[1,2], such a conclusion is known to be erroneous. It is conceivable that the UDPG-glycogen transferase may exist partly in an inactive form in skeletal muscle, although no such finding has been reported. If such be the case, it must be emphasized that the data in this paper do not give any information about changes in the amount of such a protein in pyridoxine deficiency. Thus, the nutritional experiments described here do not afford conclusive evidence against a role of pyridoxal-5′-phosphate in the activity of UDPG–glycogen transferase. However, the effect of B_6 deficiency on total phosphorylase activity is explicable in the light of knowledge of the structure of the enzyme.

NOTE ADDED IN PROOF

The UDPG-glycogen transferase activity of the normal rat gastrocnemius, excised and homogenized as described above, is increased only 32 % by assaying the enzyme in the presence of $5 \cdot 10^{-3}$ *M* glucose-6-phosphate The concentration of glucose-6-phosphate plus fructose-6-phosphate in the standard transferase assay, due to the endogenous presence of these esters in the homogenate itself, is only $1 \cdot 10^{-5}$ *M*.

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REFERENCES

[1] T. BARANOWSKI, B. ILLINGWORTH, D. H. BROWN AND C. F. CORI, *Biochim. Biophys. Acta*, 25 (1957) 16.

[2] C. F. CORI AND B. ILLINGWORTH, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 547.

[3] E. E. SNELL, *Vitamins and Hormones*, 16 (1958) 77.

[4] W. F. H. M. MOMMAERTS, B. ILLINGWORTH, C. M. PEARSON, R. J. GUILLORY AND K. SERAYDARIAN, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 791.

[5] R. SCHMID, P. W. ROBBINS AND R. R. TRAUT, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1236.

[6] R. HAUK, B. ILLINGWORTH, D. H. BROWN AND C. F. CORI, *Biochim. Biophys. Acta*, 33 (1959) 554.

[7] R. HAUK AND D. H. BROWN, *Biochim. Biophys. Acta*, 33 (1959) 556.

[8] B. ILLINGWORTH, H. S. JANSZ, D. H. BROWN AND C. F. CORI, *Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 1180.

THE KETOKINASE ACTIVITY OF THE INTESTINAL MUCOSA*

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SUMMARY

The ketokinase activity of homogenates of intestinal mucosa has been found to involve two enzymes, a heat-stable ketokinase which has a high affinity for fructose and sorbose and a heat-labile ketokinase which shows low affinity for both ketoses. The ketoses appear to be phosphorylated by both enzymes in the 1 position.

The heat-stable ketokinase has properties similar to the liver ketokinase and its presence in the intestinal mucosa is compatible with the hypothesis that the mechanism of the transformation of fructose into glucose that takes place in the intestinal mucosa might be essentially the same that operates in liver.

INTRODUCTION

Phosphorylating activities specific for ketoses have been found to occur in liver and muscle. CORI *et al.* obtained preparations of muscle and liver that phosphorylate fructose but are inactive with glucose[1]. The ketokinase activity of the muscle preparation was subsequently shown to be due to the action of phosphofructokinase[2]. This enzyme has low affinity for fructose and sorbose and phosphorylates both free sugars in the 1 position. HERS, however, has isolated a ketokinase from liver which has high affinity for fructose and sorbose[3,4]. He has elucidated the mechanism by which fructose is transformed into glucose in liver. The first step in the chain of events is the phosphorylation of fructose by ketokinase to produce fructose-1-phosphate[5].

The observation that sorbose is phosphorylated by homogenates of rat intestinal mucosa was interpreted in this laboratory as indicating the presence of a ketokinase[6]. Our efforts were directed consequently towards the identification of such an activity. The evidence reported in the present paper shows that the intestinal mucosa possesses two enzymes capable of phosphorylating fructose as well as sorbose. One of them, a heat-stable ketokinase has been purified and the study of its properties has revealed a close similarity with liver ketokinase.

SALOMON AND JOHNSON[7] have proposed recently that the transformation of

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Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris-(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; NAGA, N-acetylglucosamine.

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fructose into glucose which takes place when fructose is transported across the intestinal tissues may follow the same pathway as that of liver. Our findings provide evidence for the presence in intestinal mucosa of one of the enzymes that would be required for such a mechanism to operate in intestinal tissue. It can not be decided at present, however, whether or not this is the physiological role of the intestinal heat-stable ketokinase.

MATERIALS AND METHODS

Glucose-free fructose was prepared by recrystallization from 80% methanol of a commercial fructose (Pfanstiehl). L-Sorbose was purchased from Merck and D-arabinose from Pfanstiehl. D-Mannoheptulose was a gift from Dr. N. K. RITCHMYER. ATP and ADP were obtained from Sigma Chemical Co. as the disodium salts.

Rat-mucosa homogenates

Fed albino rats weighing 150 to 200 g were stunned, decapitated and exanguinated, the upper two thirds of the small intestine were flushed with ice cold saline, opened lengthwise and the mucosa was scrapped off with a spatula. After weighing the mucosa was homogenized for 2 min in a Waring microblendor with 2 volumes of $0.005\,M$ Versene neutralized to pH 7. Further fractionation of the homogenate will be described in the experimental section. The upper part of the small intestine of a horse was used when larger amounts of starting material were needed (purification procedure).

Measurement of the ketokinase activity

Most of the experiments were carried out as follows: small tubes containing ketose, ATP-Mg ($1.5 \cdot 10^{-2}\,M$), NaF ($2.5 \cdot 10^{-2}\,M$), Tris–maleate buffer ($1 \cdot 10^{-1}\,M$, pH 6.5), KCl ($4 \cdot 10^{-1}\,M$) and an appropriate amount of enzyme were incubated at 30° for varying lengths of time. The reaction was stopped by the addition of either 5 ml of 5% trichloroacetic acid or equal volumes of $0.3\,N$ $Ba(OH)_2$ and $ZnSO_4$. The methods used to follow the reaction were: (a) substrate disappearance, (b) esterified ketose formation, (c) non-nucleotidic acid-labile esterified phosphorus formation and (d) the photometric indicator method (see ref. 6, 8 and EXPERIMENTAL). Ketoses were determined by the method of ROE[9] or DISCHE[10]. The method of FISKE AND SUBBAROW[11] was used to analyze inorganic phosphorus. The total phosphorus was determined by analysis of the inorganic phosphorus after wet ashing with sulfuric and nitric acids. Protein was determined by the method of LOWRY *et al.*[12].

EXPERIMENTAL

Preliminary work

L-Sorbose was used as a specific substrate for the determination of the ketokinase activity in homogenates of rat intestinal mucosa that contain, in addition, a hexokinase. The hexokinase was assayed with glucose as substrate. The homogenate was centrifuged at 14,000 × *g* for 15 min and the phosphorylating activities of the supernatant were tested. Sorbose is phosphorylated 5 times faster than glucose by the supernatant while the phosphorylation of glucose by the whole homogenate is faster than that of sorbose (Table I). The assumption that sorbose is not a substrate for hexokinase is confirmed by the lack of inhibition observed when the phosphorylation of sorbose was carried out in the presence of an equimolar concentration of NAGA.

TABLE I

THE KETOKINASE ACTIVITY OF THE INTESTINAL MUCOSA; REMOVAL OF HEXOKINASE

The homogenate prepared as described in the METHODS section was centrifuged at 14,000 × *g* for 15 min. The names Supernatant II and Supernatant I correspond to the supernatants obtained when the homogenate was adjusted to pH 4.5 with 0.1 *M* HCl prior to centrifugation and when the acidification was omitted, respectively. Supernatant II was adjusted to pH 6.5 immediately after centrifugation. Supernatant I was aged by incubation at 30° for 1 h (at pH 7.5). The phosphorylating activities (µmoles of sugar phosphorylated in 15 min at 30° by 1 ml of each preparation) were determined by the esterified sugar formation method[6]. The amount of sugar in the precipitate obtained with Ba-alcohol was analyzed by the ROE's method for ketoses[9] and glucose by the method of SOMOGYI[13]. The incubation mixture was analogous to the one described in the METHODS section except that the pH was 7.5, the Mg-ATP ratio was 0.5 and the KCl was omitted.

Sugar	*0.1 M sorbose*	*0.1 M glucose*	*0.1 M sorbose + 0.1 M NAGA*
Homogenate			
Phosphorylating activity	1.5	6.2	1.5

Sugar	*0.1 M sorbose*	*0.1 M glucose*	*0.1 M glucose + 0.1 M sorbose*
Supernatant I fresh (protein 14, mg/ml)			
Phosphorylating activity	1.0	0.2	1.2

Sugar	*0.1 M sorbose*	*0.1 M glucose*
Supernatant I aged		
Phosphorylating activity	0.5	0.0

Sugar	*0.1 M sorbose*	*0.1 M fructose*	*0.1 M fructose + 0.1 M NAGA*
Supernatant II (protein 5, mg/ml)			
Phosphorylating activity	0.8	2.0	2.0

On the other hand, the implication of a ketokinase activity different from the hexokinase, as responsible for the phosphorylation of sorbose is justified by the fact that the relative rates of phosphorylation of sorbose and glucose vary from one preparation to another. The aging of the supernatant I resulted in the loss of the ability to phosphorylate glucose while the preparation was still able to phosphorylate sorbose. The absence, however, of significative amounts of hexokinase in a preparation containing most of the original ketokinase activity was only achieved if the homogenate was acidified to pH 4.5 prior to centrifugation. The resulting supernatant (supernatant II) contains 80 % of the ketokinase activity recovered when the acidification is omitted (supernatant I) with a specific activity twice as high. Supernatant II phosphorylates fructose and no significative contribution of hexokinase to this phosphorylation could be detected since an equimolar concentration of NAGA did not result in any inhibition.

At all concentrations tested fructose was phosphorylated about 2.5 times faster than sorbose (Table II). In the experiment described in Table II and all subsequent experiments the incubation mixture contained ATP-Mg in a ratio of 1 and 0.4 *M* KCl since they were found to result in maximum ketokinase activity. Under these optimal conditions phosphorylation of 0.1 *M* sorbose by the supernatant I was approximately of the order of 2.0 μmoles of sugar phosphorylated in 15 min at 30° by 1 ml of supernatant. The phosphorylation of 0.1 *M* mannoheptulose, which is 2.9 times slower than that of 0.1 *M* sorbose, could be established only under the optimal conditions.

TABLE II

THE KETOKINASE ACTIVITY OF THE INTESTINAL MUCOSA; EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF PHOSPHORYLATION OF FRUCTOSE AND SORBOSE

The activity was assayed with the standard incubation mixture described in the METHODS section and by the esterified ketose formation method[7]. The enzymic preparation used was the supernatant obtained by centrifugation at 14,000 × *g* for 15 min of the homogenate adjusted to pH 4.5 (supernatant II).

	Ketokinase activity	
Sugar concentration M	*Sorbose*	*Fructose* *
0.02	1.2	3.2
0.04	1.4	3.5
0.1	1.5	3.8
0.4	1.9	4.0

* μmoles of sugar phosphorylated in 15 min at 30° by 1 ml of enzymic preparation.

The ketokinase activity was determined indiscriminately by esterified ketose formation or by esterified phosphorus formation and in many instances by both and the complete agreement observed indicates that the product of the action of the ketokinase on sorbose and fructose is the ketose monophosphate. Moreover, the rate of acid hydrolysis of the sorbose monophosphate produced corresponds to that of sorbose-1-phosphate. The ester formed when 0.1 *M* sorbose was used, was hydrolyzed at the following rate: 40 % of the esterified phosphorus was hydrolyzed in 11 min at 100° in 0.1 *M* sulfuric acid and 85 % of it in 1 h.

Thermal fractionate inactivation

The heat inactivation of the ketokinase activity was studied using the supernatant obtained by centrifugation of the homogenate at 14,000 × *g*. The comparison of the rates of inactivation at different temperatures provided strong evidence for the presence of two enzymes with ketokinase activity in rat intestinal mucosa. The values obtained (Table III) indicate that the rate of inactivation of the ketokinase activity increases very sharply with an increase in temperature within the range of 50 to 60° and the range of 75 to 85°. The rate of thermal inactivation is independent from the temperature, between 60 and 75°. These results can be interpreted assuming that two ketokinases are present, one which is completely inactivated by 5 min of heating at 70°, and another which is not appreciably inactivated at temperatures

TABLE III

THERMAL FRACTIONATE INACTIVATION

Aliquots of supernatant I were used. They were heated at different temperatures for 5 min. Immediately after heating they were transferred to an ice bath. For the assay of the residual phosphatase and ketokinase activities the aliquots were adjusted to the same volume, the contents of the assay mixture were added (with 0.2 *M* sorbose, see the METHODS section) and except several unincubated controls, they were incubated at 30° for different lengths of time. The total volume was 1.1 ml in experiment I and 1.25 ml in experiment II. The reaction was stopped by the addition of 5 ml of 5 % TCA. After treatment with Norit A charcoal and filtration, aliquots of the filtrates were used to determine inorganic phosphorus before and after 1 h of hydrolysis in 0.1 *N* sulfuric acid at 100°. The difference in inorganic phosphorus between controls and incubated tubes was used to calculate the phosphatase activity in each tube (in separate experiments it was found that the appearance of inorganic phosphorus corresponds essentially to enzymic ATP hydrolysis). The difference in esterified phosphorus (inorganic phosphorus after acid hydrolysis minus inorganic phosphorus before acid hydrolysis) between controls and incubated tubes served to calculate the ketokinase activity in the same tubes.

Thermal inactivation (5 min of heating)		*Assay of the residual activity*		*Ketokinase activity (μmoles of phosphorus esterified/ml of extract/15 min)*	*Phosphatase activity (μmoles of incrganic phosphorus formed/ml of extract/15 min)*
Expt.	*Temperature*	*Extract used (ml)*	*Time of incubation (min)*		
I	—	0.2	60	2.0	3.75
I	50	0.2	60	2.25	2.1
II	50	0.2	60	1.95	3.0
I	55	0.2	90	1.6	1.15
I	60	0.5	90	1.0	0.65
II	60	0.5	60	0.9	0.5
I	65	0.5	90	0.8	0.4
I	70	0.6	120	0.95	0.3
II	70	0.5	90	0.9	0.15
I	75	0.6	120	0.95	0.05
II	75	0.5	90	0.95	0.02
II	80	0.6	120	0.1	0.02
II	85	0.8	120	0.0	0.02

below 75° but is completely inactivated by 5 min of heating at 85°. The names heat-labile ketokinase and heat-stable ketokinase will be subsequently used in this paper to distinguish these two enzymes. We concluded also from these data that by means of a short heating at a suitable temperature, a preparation of heat-stable ketokinase could be obtained that was free from phosphatase and heat-labile ketokinase.

Purification of the heat-stable ketokinase

A survey of the ketokinase activity content in the intestinal mucosa of several species of mammals was carried out. Dog, sheep, cow and horse intestinal mucosa was found to contain approximately the same activity. Horse small intestine was chosen as starting material because the mucosa can be removed easily and also a single long piece of small intestine provided enough material to carry out the purification. The procedure for the purification of the heat-stable ketokinase consists essentially of a short heating at 70° followed by ammonium sulfate fractionation in order to concentrate the enzyme (Table IV).

In each step of the purification, the ketokinase activity was assayed by the esterified phosphorus formation with sorbose as substrate. In step 1 the ratio between the rates of phosphorylation of sorbose at $2 \cdot 10^{-1}\,M$ and $5 \cdot 10^{-3}\,M$ initial concen-

TABLE IV

PURIFICATION OF THE HEAT-STABLE KETOKINASE FROM HORSE INTESTINAL MUCOSA

Starting material: 460 g of scrappings of intestinal mucosa from the small intestine of a horse. The ketokinase activity is given in μmoles of 0.005 M sorbose phosphorylated by 1 ml of extract in 15 min. Step 1, preparation and centrifugation of the homogenate. A piece of small intestine (about 6 meters long) was taken from a slaughtered horse and chilled in ice. The contents were washed off with cold water and the intestine was opened lengthwise. The mucosa was removed carefully with a spatula in the cold room. The scrappings (460 g) were homogenized with 2 volumes of 0.005 M Versene in a Waring type blendor for 2.5 min. The homogenate was kept overnight at —10°. After thawing it was centrifuged at 14,000 $\times$ g for 15 min. The supernatant was removed and the precipitate discarded. Step 2, heating. Fractions of 100 ml of the supernatant (900 ml) were heated in a 250-ml Erlenmeyer placed in a water bath with continuous stirring. The temperature of the fluid rose up to 70° in 2 min and was maintained at 70° for 3 additional minutes. The Erlenmeyer was then cooled with ice water and the fluid was kept frozen overnight at —18°. After thawing the resulting clotty suspension was centrifuged at 14,000 $\times$ g for 15 min. The supernatant was removed and the precipitate discarded. Step 3, fractionation with ammonium sulfate. Solid ammonium sulfate (170 g) was added to the supernatant (870 ml) to give a 33% saturated solution. After allowing 12 h at 2° for precipitation it was centrifuged at 10,000 $\times$ g for 10 min. The precipitate was discarded and to the supernatant (920 ml) solid ammonium sulfate was added (98 g) to give a 50% saturated solution. This was left overnight at 2° and centrifuged as before. The precipitate was suspended in 0.005 M Versene and dialyzed against renewed water for 4 h.

Step	*Volume ml*	*Ketokinase activity μmoles/ml/15 min*	*Protein mg/ml*	*Purification*	*Recovery %*
1	900	1	13		
2	870	1	4	× 3.3	96
3	11	28	34	× 11.7	33

tration varied from one preparation to another from 2.0 to 3.25. In step 2 the ratio was consistently 2.0. These values indicate the presence in some step 1 preparations of a rather unstable ketokinase which was destroyed completely by heating at 70° for 5 min. This heat-labile ketokinase has apparently a low affinity for sorbose since the rate of phosphorylation at $5 \cdot 10^{-3}$ M sorbose was the same before and after heating.

The values obtained for the ketokinase activity with low sorbose concentration were used to ascertain the purification of the heat-stable ketokinase attained in each step of the procedure. It was assumed that with low substrate concentration the activity measured was exclusively due to the action of the heat-stable ketokinase even when both ketokinases are present, *i.e.* in the preparation obtained in step 1. The procedure presents a certain degree of variability in step 3 but always yielded in this step a preparation very suitable for the study of the properties of the heat-stable ketokinase. This preparation contains a relatively high ketokinase activity which is stable for months at —20°, and it is free from phosphatase, hexokinase and heat-labile ketokinase.

Properties of the heat-stable ketokinase

Affinity: The phosphorylation of fructose and sorbose by the heat-stable ketokinase was followed with the photometric indicator method[8].

The relative rates of phosphorylation of fructose and sorbose at different concentrations were determined by taking the reciprocal of the time required for a given decrease in O.D. The results (Fig. 1) showed the K_m for fructose to be approx. $7 \cdot 10^{-4}\, M$, the K_m for sorbose to be approx. $5 \cdot 10^{-3}\, M$ and the ratio V_{max} sorbose/V_{max} fructose to be approx. 2.0.

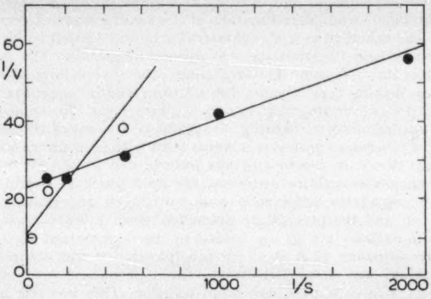

Fig. 1. Phosphorylation of fructose and sorbose at different concentrations by the heat-stable ketokinase. LINEWEAVER AND BURK representation; v is expressed in arbitrary relative values obtained by the photometric indicator method and S are molar concentrations. ○ ○ ○, sorbose; ● ● ●, fructose.

The phosphorylation of fructose was not appreciably affected by changing the concentration of ATP from 3 to $15 \cdot 10^{-3}\, M$ (Table V). This behaviour indicates that the affinity for the donor ATP may be as high as has been observed with other kinases[3,8]. The addition of ADP inhibits markedly the heat-stable ketokinase. The inhibition appears to be relieved at least partially by increasing the ATP concentration. The results obtained do not constitute enough evidence, however, to conclude that the inhibition by ADP is strictly competitive as it has been proved to be with brain hexokinase[8]. Liver ketokinase is also inhibited by ADP although the available reports are not in agreement on whether the inhibition is competitive[14] or noncompetitive[15].

Reaction product: The product accumulated during the phosphorylation of sorbose by the heat-stable ketokinase was isolated as described in Table VI. The conclusion that the esterified sugar is sorbose monophosphate is supported by the results of the analysis of phosphate and ketose. That the product is sorbose-1-phosphate is indicated by the rate of acid hydrolysis of the phosphate group.

TABLE V

INHIBITION BY ADP OF THE PHOSPHORYLATION OF FRUCTOSE BY THE HEAT-STABLE KETOKINASE

Two series of tubes were prepared containing in 1.9 ml, 2 µmoles of fructose, 100 µmoles of Tris-maleate buffer, pH 6.5, 800 µmoles of KCl, 30 µmoles of $MgSO_4$ and the appropriate amounts of ATP to give the initial concentrations of 15, 7.5 and $3.7 \cdot 10^{-3}\, M$. The tubes in the one series contained, in addition, 15 µmoles of ADP. To each tube 0.1 ml of the preparation obtained in step 3 of the purification procedure (diluted 8 times) was added. Except the unincubated controls, all the tubes were incubated at 30° for 30 min. They were deproteinized with $Ba(OH)_2$ and $ZnSO_4$ and ketose was determined by the method of DISCHE[10]. The phosphorylation of fructose was estimated by substrate disappearance.

ATP, average molar concentration × 10^3	11.5	11.9	5.75	5.9	2.8	3.0
ADP, average molar concentration × 10^3	0.21	6.1	0.25	6.1	0.2	6.0
Fructose phosphorylated (referred to 1 ml)	0.42	0.18	0.50	0.15	0.41	0.11

TABLE VI

ANALYSIS OF THE PRODUCT OF THE REACTION OF PHOSPHORYLATION OF SORBOSE BY THE HEAT-STABLE KETOKINASE

To a tube containing in a volume of 1.8 ml, 100 μmoles of sorbose; 600 μmoles of Tris–maleate buffer, pH 6.5; 800 μmoles of KCl; 75 μmoles of ATP and 75 μmoles of $MgSO_4$, 0.2 ml of the preparation obtained in the step 3 of the purification procedure were added. It was incubated at 30° for 2 h. 8 ml of 5% TCA were added, it was shaken with 1.5 g of norit A charcoal for 15 min and filtered. To 5 ml of the filtrate, phenolphtalein and 0.2 ml of 0.1 M barium acetate were added. After 5 min in the cold it was centrifuged and the supernatant was decanted. To 5 ml of the supernatant 20 ml of 96% alcohol were added and after 45 min in an ice bath, it was centrifuged. The precipitate was dissolved in 0.35 ml of 0.1 N HCl. 0.4 ml of water and 0.1 ml of 0.5 M K_2SO_4 were added. It was centrifuged, checked for excess barium and then neutralized and adjusted to 3 ml with water. Suitable aliquots were taken for the different determinations.

	μmoles	
Ketose	36	
Carbohydrate	33	
Total esterified phosphorus	36	
Acid hydrolysis in 0.1 N sulfuric acid at 100°		*% of total*
Phosphorus hydrolyzed in 5 min	6.6	15
10	12	33
60	33	90

Specificity: The phosphorylation of sorbose is inhibited by fructose. The rate of phosphorylation of an equimolar mixture of 0.05 M fructose and sorbose determined by the photometric indicator method[8] was about 40% of the rate of sorbose phosphorylation and approximately equal to the rate of fructose phosphorylation at the same concentration. The phosphorylation of 0.01 M sorbose was not inhibited by the presence of a concentration of D-arabinose 10 times higher. This result is in contrast with the pattern of specificity of brain hexokinase. Hexokinase is inhibited by pentoses which differ from the corresponding hexose substrate by the absence of the carbon on which the phosphate should be attached. In the case of the heat-stable ketokinase, it appears that the sugar must be a ketose in order to combine with the enzyme. It was not possible to detect with the photometric indicator method[8] any phosphorylation of 0.016 M mannoheptulose by the heat-stable ketokinase. The supernatant obtained by centrifugation of the homogenate phosphorylates 0.1 M mannoheptulose about 1/3 as fast as 0.1 M sorbose (see preliminary work). This indicates that mannoheptulose is a good substrate for the heat-labile ketokinase but not for the heat-stable ketokinase. This difference in specificity with respect to mannoheptulose between the two intestinal ketokinases has its parallelism in liver and muscle ketokinases. KUYPER[14] found that mannoheptulose is not phosphorylated by liver ketokinase and VILLAR-PALASI AND SOLS[2] have reported that mannoheptulose is substrate for muscle phosphofructokinase.

DISCUSSION

Because of the possible implication in the mechanism of transport of sugars from the intestine, attention has been directed towards the transformation of fructose into glucose that takes place in the intestinal mucosa[16–18]. SOLS AND ALVARADO* have searched insistently for the possibility of a direct transformation of fructose into glucose by homogenates of rat intestinal mucosa and have failed to detect any isomerization. Recently SALOMON AND JOHNSON[7] have proposed that the transformation of fructose into glucose in the intestine may follow the same path as that in liver and they have reported evidence in favor of this hypothesis. Our experiments show the presence in the intestinal mucosa of a heat-stable ketokinase which is very similar in its properties to the liver ketokinase. That the role of the heat-stable ketokinase in the intestinal mucosa is analogous to the function of liver ketokinase in the transformation of fructose into glucose, although suggestive, remains to be proved. Evidence has been obtained indicating that in the intestinal mucosa in addition to a heat-stable ketokinase there is a heat-labile enzyme which also phosphorylates ketoses. In homogenates of rat intestinal mucosa this latter enzyme is responsible for approx. 50 % of the total phosphorylation of 0.2 M sorbose. Both heat-stable and heat-labile ketokinases phosphorylate fructose and sorbose in the 1 position. Aside from their different heat-stability they differ in two main characteristics: (a) The heat-labile ketokinase has a very low affinity for fructose and sorbose in contrast with the high affinity of the heat-stable ketokinase for both sugars and (b) the ratio of the relative maximum rates of phosphorylation of sorbose and fructose by the heat-stable ketokinase is 2.0 and approximately 0.5 for the heat-labile ketokinase. It should be emphasized that the properties of the ketokinase activity present in homogenates and crude extracts of intestinal mucosa differ markedly from the properties of liver ketokinase. In fact it resembles the muscle ketokinase activity. Only after purification the presence of a ketokinase similar to the liver enzyme becomes evident. By analogy with the observations of VILLAR-PALASI AND SOLS[2] on the ketokinase activity of muscle it is suggested that the heat-labile ketokinase of the intestinal mucosa may be a marginal activity of the intestinal phosphofructokinase. Like muscle phosphofructokinase the heat-labile ketokinase of the intestinal mucosa has low affinity for fructose and sorbose and phosphorylates mannoheptulose which is not a substrate for the intestinal heat-stable ketokinase nor the liver ketokinase[15].

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* A. SOLS AND F. ALVARADO, unpublished results.

REFERENCES

[1] M. W. SLEIN, G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 186 (1950) 763.

[2] C. VILLAR-PALASI AND A. SOLS, *Bull. soc. chim. biol.*, 339, *Suppl.* 2, (1957) 71.

[3] H. G. HERS, *Biochim. Biophys. Acta*, 8 (1952) 415.

[4] H. G. HERS, *Biochim. Biophys. Acta*, 8 (1952) 424.

[5] H. G. HERS AND T. KUSAKA, *Biochim. Biophys. Acta*, 11 (1953) 427.

[6] A. SOLS, *Biochim. Biophys. Acta*, 19 (1956) 144.

[7] L. L. SALOMON AND J. E. JOHNSON, *Arch. Biochem. Biophys.*, 82 (1959) 179.

[8] R. K. CRANE AND A. SOLS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1955, p. 277.

[9] J. H. ROE, J. H. EPSTEIN AND N. P. GOLDSTEIN, *J. Biol. Chem.*, 178 (1949) 839.

[10] Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 192 (1951) 583.

[11] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.

[12] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

[13] M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19.

[14] CH. M. A. KUYPER, Thesis, Nijmegen, 1955.

[15] R. E. PARKS, JR., E. BEN-GERSHOM AND H. A. LARDY, *J. Biol. Chem.*, 227 (1957) 231.

[16] W. A. DARLINGTON AND J. H. QUASTEL, *Arch. Biochem. Biophys.*, 43 (1953) 194.

[17] J. Y. KIYASU AND I. L. CHAIKOFF, *J. Biol. Chem.*, 224 (1957) 935.

[18] T. H. WILSON AND T. N. VINCENT, *J. Biol. Chem.*, 216 (1955) 851.

ON THE MECHANISM OF THE REACTION OF THE REDUCED LACCASE WITH OXYGEN

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SUMMARY

1. The kinetics of the reaction of reduced laccase and oxygen have been studied by spectrophotometry and magnetometry, and the results showed that a Cu^+–O_2 complex does not exist in the reaction.

2. The second order velocity constant of the above reaction was found to be of comparable value to that of the peroxidase–H_2O_2 reaction. The fact that the velocity constant does not change at different oxygen concentrations supports statement 1 above.

3. The velocity constant is not affected by pH change as long as the enzyme does not denature. For the laccase–O_2 reaction, no copper-linked acid group was found in the laccase molecule.

INTRODUCTION

The following reaction mechanism has been proposed for laccase[1]:

$$2\,Cu^+ + \tfrac{1}{2}\,O_2 + 2\,H^+ \longrightarrow 2\,Cu^{++} + H_2O \qquad (1)$$

$$2\,Cu^{++} + \text{hydroquinone} \longrightarrow 2\,Cu^+ + p\text{-quinone} + 2\,H^+ \qquad (2)$$

Two points are clear experimentally concerning reaction (1): first, the oxidation from cuprous to cupric is spectrophotometrically[2] and magnetometrically[3, 4] operative; second, the stoichiometry of the reaction is as shown in eqn. (1)[1]. These results agree with each other in the economy of the transferred electron, under the condition that, in the reaction, molecular oxygen is reduced to water, not hydrogen peroxide. However, these static methods leave one question unsolved: is a transient enzyme–substrate complex, Cu^+–O_2 formed in this reaction before the electron is transferred from copper to oxygen?

Kinetic observations by magnetometric and spectrophotometric approaches under the same conditions may be able to provide a solution to the problem, and some evidence obtained by these methods is presented in this paper.

MATERIALS AND METHODS

Laccase: Laccase was purified from the latex of the Chinese lacquer tree (*Rhus vernicifera*), obtained from Ken Shii, China. The procedures of extraction and purification of the enzyme were essentially the same as those described previously[2].

Ascorbate: Ascorbic acid, purchased from Eastman Organic Chemicals, was freshly neutralized and used for the experiments.

Measurement of oxygen uptake: The platinum microelectrode which was developed for the polarographic estimation of dissolved oxygen[5,6], improved by CHANCE for further biochemical applications[7], was used to record the uptake of oxygen in the laccase reaction.

Spectrophotometric measurements: A double beam spectrophotometer designed by CHANCE[8] provided with a flow apparatus of 10 mm optical path was used to record the change of O.D. of the reaction mixtures. One of the two syringes of the flow apparatus was filled with a laccase solution containing ascorbate, and the other with an oxygen solution of known concentration, obtained by bubbling buffer solution with a gas mixture. The syringe plungers were pushed down and the change of the O.D. of the mixed solution at 615 mμ during flow (accelerated flow method) and after the flow was stopped (stopped flow method) was recorded. The time during flow was calculated from the flow velocity trace. The details of this procedure are given by CHANCE[9]. 500 mμ was used as the reference wavelength.

Magnetometric measurements: BRILL *et al.*[10] developed a magnetic susceptometer of the Rankine balance type for sensitive volume paramagnetic susceptibility measurements. In the flow apparatus[11], combined with the susceptometer, the solution in the main pump-1, after flowing through the half cell-1 and reaching the mixing chamber, is mixed with a solution from the secondary pump; the mixture then flows through the half cell-2. The flow rate is maintained at a constant value during each flow. According to the change of the magnetic susceptibility of the solution caused by mixing with the secondary solution, the magnet which is hung between the two half cells moves toward the cell of the larger paramagnetism, the movement being detected photoelectrically, and the signal obtained being amplified and recorded on chart paper. The signal strength in the record is proportional to the change of magnetic susceptibility of the solution. Because the mixing ratio is as small as 1:82.6, the slight change in the concentration of the components in the main solution can be ignored. Usually, flow noise was observed when the flow was started. After a few seconds, when the noise had ceased, the secondary pump was pushed and the solution in it mixed into the main solution, and the change of magnetic susceptibility recorded. The sensitivity of the susceptometer was calibrated with a $NiCl_2$ solution as standard.

Measurement of laccase concentration: The concentration of laccase was determined by the molecular extinction coefficient of laccase reported previously[2], using a Beckman DU spectrophotometer.

Experimental conditions: All the experiments were performed in $M/30$ phosphate buffer of pH 7.0 at 25° unless notified. The pH was checked with a glass electrode pH meter made by Analytical Measurements Inc.

RESULTS

Kinetic measurements of laccase reaction by magnetometry and spectrophotometry

Magnetometry: An anaerobic solution of the reduced laccase with an excess amount of ascorbate was placed in the main pump of the flow apparatus of the susceptometer, and a saturated solution of oxygen at one atmospheric pressure was placed in the secondary pump. The laccase concentration was adjusted so that the amount of the oxygen mixed in is in stoichiometric proportion to laccase. A typical record from this experiment is shown in Fig. 1. By changing the speed of flow and the concentration of ascorbate, the kinetic curves of the magnetic susceptibility change of laccase solution can be obtained (see Fig. 2). These curves show a rapid oxidation of copper by molecular oxygen followed by slow reduction by ascorbate. As will be shown in a later section of this paper, the second order velocity constant of the reaction of reduced laccase and oxygen (k_1) is $2.8 \cdot 10^6\ M^{-1}\ \text{sec}^{-1}$.

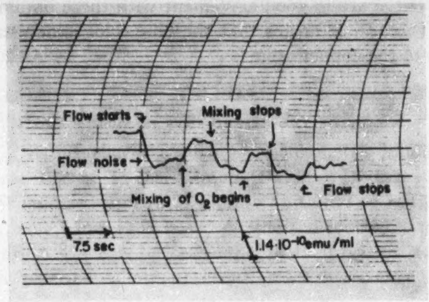

Fig. 1. A magnetometric record of the laccase reaction obtained by the susceptometer at 22°. Initial concentrations of laccase, oxygen and ascorbate are 60 μM (on the basis of copper concentration), 15 μM and 9.8 mM respectively.

$$\text{Reduced laccase} + O_2 \xrightarrow{k_1} \text{oxidized laccase} + O_2^{----} \qquad (3)$$

so the amount of oxygen, remaining unreacted in the reaction mixture after a given period, can be calculated. As molecular oxygen itself has a paramagnetic susceptibility of $3390 \cdot 10^{-6}$ emu/mole at 20° (see ref. 12), the susceptibility of the remaining oxygen must be subtracted from the overall increase of the paramagnetic susceptibility. The plots in Fig. 2 show the corrected values. The curve I in Fig. 2 shows that the maximum change in magnetic susceptibility of the laccase copper corresponds to the cuprous to cupric change. This result is coincident with the previous experiment with a Gouy magnetic balance[3]. In these curves, no evidence appeared to indicate that a transient Cu^+–O_2 complex exists.

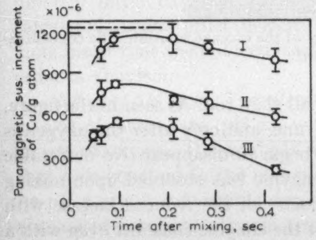

Fig. 2. Kinetics of the laccase reaction by magnetometry at pH 7.0. ——·——, represents the theoretical value of the magnetic susceptibility of cupric copper at 22°. For details, see Table I. Points are shown with root-mean-square error.

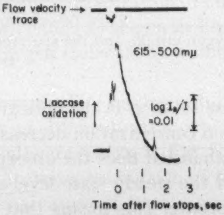

Fig 3. A stopped flow record of the laccase reaction by spectrophotometry. For details, see Table I.

Spectrophotometry: The spectrophotometric record of the reaction under the same experimental condition as that of curve III of Fig. 2 is shown in Fig. 3. This record is a stopped flow experiment. The observed maximum value of cupric copper ($[Cu^{++}]_{max}$) and $t_{\frac{1}{2}\mathrm{off}}$* of this curve are in good agreement with those obtained from magnetic measurement (see Table I). It means that the blue color is necessarily accompanied by the cupric state of the copper atom.

TABLE I

SUMMARY OF THE EXPERIMENTS IN FIGS. 2 AND 3

Experiment	*[Laccase]** *µM*	*$[O_2]$*** *µM*	*Temperature*	*[Ascorbate]* *mM*	*$[Cu^{++}]_{max}$** *µM*	*$t_{\frac{1}{2}\mathrm{off}}$* *(sec)*
Magnetometric I***	60	16	21	1.3	57	—
Magnetometric II	60	15	22	9.8	40	—
Magnetometric III	60	15	22.5	20.0	28	0.35
Spectrophotometric	60	15	25	20.0	27	0.4

* On the basis of coppper concentration.
** Initial concentration.
*** Magnetometric I, II and III correspond to the curves I, II and III in Fig. 2.

Velocity constant and mechanism of the reaction of the reduced laccase and oxygen

Preliminary experiment: By using the platinum microelectrode and the double beam spectrophotometer, a simultaneous measurement of the change of oxygen concentration and O.D. at 615 mµ upon mixing ascorbate into the air-saturated laccase solution was undertaken (Fig. 4). In this experiment, ascorbate was added to the laccase solution in the optical cuvette by a small glass rod. As seen in the figure, the oxygen concentration decreases linearly with time, and only after the oxygen is almost exhausted does the absorption at 615 mµ begin to disappear. No detectable change of the steady state level of the oxidized enzyme was observed upon mixing with ascorbate. This means that the reaction velocity of the reduced laccase with oxygen is fast enough to keep the oxidized level of the enzyme constant even with a very low level of oxygen.

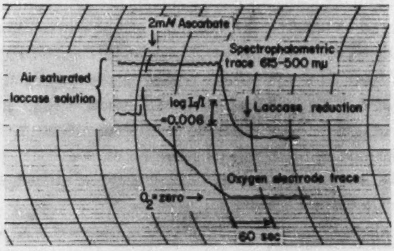

Fig. 4. Oxygen uptake and the spectrophotometric change of the laccase solution. The concentration of laccase is 6 *µM*.

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* The time interval from half-maximal formation of cupric copper until its concentration has fallen to half its maximal value.

k_1 measurement by spectrophotometry: The accelerated flow method was employed for measurement of the k_1 of eqn. (3) by mixing an anaerobic solution of the reduced laccase with an oxygen solution in the flow apparatus. A typical record is shown in Fig. 5. Dilute ascorbate was used to reduce the enzyme so that complete oxidation of laccase copper was obtained when the flow stopped. In order to check the order of the reaction, the values of

$$\frac{[\text{Oxidized Laccase}]}{[\text{Total Laccase}]\,([\text{Total Laccase}] - [\text{Oxidized Laccase}])}\ (= k_1 \cdot t)$$

for the reaction of reduced laccase with a stoichiometric amount of oxygen were plotted against time (Fig. 6). In this formula [Oxidized Laccase] and [Total Laccase] are the amount of oxidized and total laccase at time t; t is the time during flow, *i.e.* time after mixing, and k_1 is based on the molecular oxygen and laccase (not copper) concentration. As can be seen, the plots drop on a straight line which indicates that the reaction follows a second order mechanism. The value obtained for k_1 is $2.8 \cdot 10^6\ M^{-1}\ \text{sec}^{-1}$ at 25° and pH 7.0. This experiment was repeated at different initial oxygen concentrations with a constant concentration of laccase. The calculated second order velocity constant did not change as shown in Fig. 7. These facts mean that the reduced laccase reacts with oxygen molecule by a simple second order mechanism.

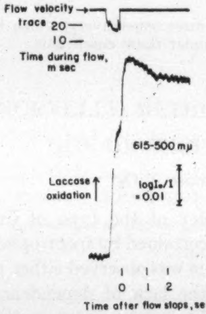

Fig. 5. An accelerated flow record of the laccase reaction by spectrophotometry. Initial concentrations of laccase, oxygen and ascorbate are 11 μM, 25 μM and 2 mM respectively.

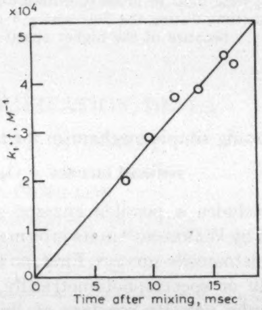

Fig. 6. Plot of $k_1 \cdot t$ against t. Initial concentrations of laccase, oxygen and ascorbate are 14 μM, 14 μM and 2 mM respectively.

Effect of pH on k_1: The effect of pH on k_1 was studied by mixing reduced laccase in 0.01 M phosphate, pH 7.0 with 0.1 M buffers of different pH's, which contain 50 μM of oxygen. The final pH was checked. Results are shown in Fig. 8. It is seen that k_1 is constant in the region of pH 4–9, and k_1 decreases outside of this region. At pH 2 or 10, irreversible decolorization and inactivation of laccase occurs in the absence of substrate at room temperature. So, this decrease of k_1 can be attributed to the denaturation of the enzyme. It can be said that any acidic group which dissociates hydrogen ion in this pH region (4–9) in laccase molecule does not affect the reaction (1) by dissociating its proton. The overall activity of laccase has the maximum at pH 7.4 (see ref. 2) and the activity decreases markedly on both sides of pH 7.4: this means that only the reduction step of laccase by substrate is pH dependent.

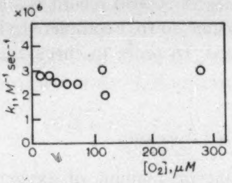

Fig. 7. Effect of the concentration of oxygen on k_1. The concentration of laccase is 20 μM. The concentration of ascorbate is 2 mM or 5 mM for experiments with O_2 concentration of under 100 μM or above 100 μM, respectively. For experiments with O_2 concentrations between 40–80 μM and 120–300 μM, flow apparatuses of 2 mm and 1 mm optical path[13] respectively were used in order to obtain higher flow speed (time during the flow was 4–8 msec and 0.8–2.0 msec respectively) which is required because of the higher speed of the reaction under these conditions.

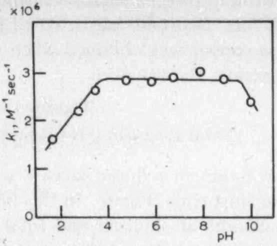

Fig. 8. Effect of pH on k_1. Glycine, acetate and the KOLTHOFF's buffers were used for pH ranges of 1.9–3, 4–5 and 6–10, respectively. The concentration of ascorbate is 2 mM.

DISCUSSION

The following simple mechanism for the oxidation of laccase

$$\text{reduced laccase} + O_2 \dashrightarrow \text{oxidized laccase} + O_2^{----} \qquad (3)$$

which excludes a possible enzyme–substrate complex of the type of $Cu^+–O_2$ as proposed by WARBURG[14] in its intermediate step, is ascertained by spectrophotometric and magnetometric surveys. First, no transient complex was observed either magnetometrically or spectrophotometrically, and second, the lack of dependency of the second order velocity constant of this reaction on the oxygen concentration means that the speed of utilization of oxygen by the reduced laccase is proportional to oxygen concentration throughout the range studied (14–280 μM).

Laccase and ascorbic acid oxidase have similar characteristics. They are both oxygen-linked oxidases with copper atoms as the prosthetic group, they are both deep blue, and both are reduced to leuco-form by ascorbic acid. THIMANN *et al.*[15] reported that the ascorbic acid oxidase has a weak affinity for oxygen. In contrast to this, in the case of laccase, the oxidation of the enzyme by molecular oxygen proceeds very quickly, and the value obtained for the second order velocity constant is comparable to that of the reaction of the formation of enzyme–substrate complex between horse radish peroxidase and hydrogen peroxide[16], which means a high affinity of laccase copper for oxygen.

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. B. CHANCE for his kind guidance throughout this work. He is indebted also to Dr. A. S. BRILL for his valuable discussions with him. The author appreciates the kindness of the Saito Co. Ltd., Osaka, for their supply of crude latex used in this work.

REFERENCES

[1] T. Nakamura, *Biochim. Biophys. Acta*, 30 (1958) 538.
[2] T. Nakamura, *Biochim. Biophys. Acta*, 30 (1958) 44.
[3] T. Nakamura, *Biochim. Biophys. Acta*, 30 (1958) 640.
[4] B. G. Malmström, R. Mosbach and T. Vänngård, *Nature*, 183 (1959) 321.
[5] P. W. Davies and F. Brink, Jr., *Rev. Sci. Instr.*, 13 (1942) 524.
[6] I. M. Kolthoff and J. J. Lingane, *Polarography*, Interscience Publishers, New York, 1952, p. 399.
[7] B. Chance, *J. Biol. Chem.*, 217 (1955) 383.
[8] B. Chance, *Rev. Sci. Instr.*, 22 (1951) 619.
[9] B. Chance, *Arch. Biochem. Biophys.*, 22 (1949) 224.
[10] A. S. Brill, H. den Hartog and V. Legallais, *Rev. Sci. Instr.*, 29 (1958) 383.
[11] A. S. Brill, H. den Hartog and V. Legallais, *Rev. Sci. Instr.*, 29 (1958) 242.
[12] P. Selwood, *Magnetochemistry*, 2nd ed., Interscience Publishers, New York, 1956, p. 246.
[13] B. Chance, *J. Biol. Chem.*, 151 (1943) 553.
[14] O. Warburg, *Heavy Metal Prosthetic Groups and Enzyme Action*, Oxford Clarendon Press, 1949, p. 181.
[15] K. V. Thimann, C. S. Yocum and D. P. Hackett, *Arch. Biochem. Biophys.*, 53 (1954) 239.
[16] B. Chance, in W. D. McElroy and B. Glass, *The Mechanism of Enzyme Action*, Johns Hopkins Press, 1954, p. 391.

NOUVELLE METHODE DE PURIFICATION DE LA GONADOTROPINE CHORIALE HUMAINE

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SUMMARY

A new method of purification of human chorionic gonadotropin

Human chorionic gonadotropin is purified up to 12,000 IU/mg, from urine of pregnant women, by a new method of preparation. After benzoic acid adsorption and extraction in an acetate buffer of pH 4.8, the method involves a fractionation with calcium in 50 % ethanol and a precipitation at the pHi. Kaolin adsorption and chromatography on Decalso columns yield a fraction at a titre of 7,000 IU/mg.

A further purification by chromatography on Dowex 2 columns or starch electrophoresis gives a final preparation of 10,000 to 12,000 IU/mg, which behaves as a homogenous substance in a physicochemical test for purity.

INTRODUCTION

L'étude des caractéristiques physico-chimiques d'une hormone protéique est subordonnée à l'obtention d'une substance pure. Aussi, les méthodes d'extraction et de purification de la gonadotropine choriale humaine se sont-elles succédées depuis trente ans.

La plupart de ces méthodes sont basées essentiellement sur deux procédés: d'une part, l'adsorption benzoïque[1], suivie de fractionnement par les solvants organiques; GURIN, BACHMAN ET WILSON[2], puis CLAESSON, HOGBERG, ROSENBERG ET WESTERMAN[3] utilisèrent ce procédé pour obtenir des hormones hautement purifiées.

D'autre part, l'adsorption sur Permutite, qui, effectuée directement à partir de l'urine, donnait à KATZMAN, GODFRID, CAIN ET DOISY[4] une substance identique à celle de GURIN *et al.*[2].

Cependant, aucune de ces méthodes n'a permis d'obtenir un produit qui titrat plus de 6,000 UI/mg, et dont la pureté ait été parfaitement démontrée.

L'hormone issue de notre méthode de purification atteint 12,000 UI/mg, et sa pureté a été montrée par plusieurs critères[5].

MÉTHODES ET RÉSULTATS

Dosage biologique

L'activité biologique a été déterminée par l'augmentation de poids des vésicules séminales[6] chez le rat mâle impubère. Les animaux, âgés de 21–23 jours et pesant de 25 à 30 g, recevaient trois injections de 1 ml en trois jours. Les fractions à titrer et le témoin étaient solubilisés dans le sérum physiologique au moment de l'injection. Les doses injectées étaient d'une à deux Unités Internationales; les animaux étaient sacrifiés le quatrième jour. En général, dix animaux étaient utilisés pour chaque fraction. Les produits correspondant à la fin des préparations ou à certains stades particulièrement importants étaient dosés par un essai quatre points.

Sources et hormone brute

La découverte par BROWNE ET VENNING[7], confirmée par EVANS, KOHLS ET WONDER[8], d'un maximum d'excrétion de la gonadotropine choriale dans l'urine, devait permettre aux chercheurs d'utiliser une matière première plus riche: l'urine recueillie aux alentours du soixantième jour de grossesse.

De telles urines ont pu être utilisées, mais en quantité relativement faible (quelques dizaines de litres): elles titraient environ 30,000 UI/l.

Les principales préparations ont été effectuées sur des mélanges d'urines recueillies entre le 60ième et le 90ième jour de grossesse: le titre variait entre 8,000 et 12,000 UI/l.

Les urines étaient stockées en chambre froide sur merthiolate.

L'extraction de l'activité gonadotrope, à partir de ces urines, était effectuée selon la méthode d'adsorption sur l'acide benzoïque de KATZMAN ET DOISY[1]. Le produit obtenu etait extrait à 4° par un tampon acétate $M/15$, de pH 4.8. Un insoluble important et pratiquement inactif était ainsi éliminé. Une précipitation par 80 % d'acetone permettait d'obtenir une hormone brute (produit P) contenant la quasi totalité de l'activité initiale.

Des exemples du produit P sont donnés dans le Tableau I.

Extraction hydro-alcoolique

Par analogie avec la technique de fractionnement des mucopolysaccharides employée par MEYER *et al.*[9,10], on pouvait tenter de séparer les mucoprotéines urinaires en se fondant sur la différence de solubilité de leurs sels de calcium. Le produit P était extrait ainsi par l'éthanol à 50 % dans un tampon acétate $M/15$, de pH 4.5, contenant une concentration molaire 0.015 M d'un sel de calcium, chlorure ou acétate. L'extraction était effectuée en trois fois, avec agitation mécanique, à 4°, par un volume total de x ml, pour une quantité 10 x mg de P. L'insoluble, pratiquement inactif était éliminé, la solution surnageante était amenée à une concentration alcoolique de 80 %: on retrouvait dans le précipité obtenu l'essentiel de l'activité initiale, ainsi qu'en témoignent les exemples rassemblés dans le Tableau I.

TABLEAU I

EXTRACTION HYDRO-ALCOOLIQUE DE LA GONADOTROPINE CHORIALE EN PRÉSENCE DE CALCIUM

UI/l d'urine	*P*				*Insoluble à 50 % d'éthanol en présence de calcium*			*Produit final*		
	Poids total	*Poids/l*	*UI/mg*	*UI (total)*	*Poids*	*UI/mg*	*UI (total)*	*Poids*	*UI/mg*	*UI (total)*
27,000	1,000	22	963	963,000	722	124	90,000	106	5,425	545,000
23,000	785	27	700	549,500	380			312	1,670	521,000
24,000	826	20	1,200	991,000	360	149	53,640	392	2,250	882,000
	760				310	53	16,430	180	2,400	432,000
14,000	1,195	12.5	960	1,147,000	625	145	90,625	430	2,250	967,000
11,500	33,800	19	550	18,600,000	20,400	100	2,040,000	7,980	1,820	14,523,000
8,500	60,000	57	135	8,100,000	52,000	20	1,040,000	5,760	1,200	6,910,000
9,500	4,000	24.5	350	1,715,000	2,966	37	109,700	1,320	1,120	1,452,000

La lecture de ce Tableau montre que l'activité de l'hormone brute P est d'autant plus élevée que l'on part d'une urine plus riche. Cependant, après cette extraction hydro-alcoolique, l'activité spécifique des produits obtenus ne varie que dans des limites assez restreintes, quelque soient la quantité et la richesse des urines utilisées.

A ce stade de la préparation, le rendement est voisin de 80 %.

Précipitation au pHi

Cette extraction hydro-alcoolique était complétée par une précipitation sélective de l'hormone à son pHi.

Le produit hormonal provenant du stade précédent était dissous, à une concentration de 1.5 %, dans une solution d'acide acétique ajustée pH 3. Un précipité, obtenu en amenant la solution à une concentration de 60 % en éthanol, contenait l'activité gonadotrope; la purification était d'environ 50 %.

Adsorption sur kaolin

Le kaolin est fréquemment utilisé pour l'extraction des gonadotropines par adsorption à partir de l'urine normale. Son emploi dans la purification de la gonadotropine choriale, à un stade relativement avancé de la préparation, n'a jamais été signalé. L'hormone était adsorbée à pH 4.5 et éluée par une solution ammoniacale de pH 11.5: 1 ml d'une suspension de kaolin à 20 % pouvait adsorber 10,000 UI. Le titre des précipités acétoniques obtenus à partir de cet éluat ammoniacal neutralisé, dépassait dans la plupart de nos préparations, 3,500 UI/mg. La purification était de 100 %, avec un rendement toujours supérieur à 90 %.

Le Tableau II donne quelques exemples de la purification obtenue par adsorption sur kaolin à partir du produit précipité au point isoeléctrique.

TABLEAU II

ADSORPTION SUR KAOLIN DU PRODUIT PRÉCIPITÉ AU pHi DE L'HORMONE

Produit adsorbé			*Produit élué*		
Poids (mg)	*UI/mg*	*UI (total)*	*Poids (mg)*	*UI/mg*	*UI (total)*
154	2,920	450,000	79	5,200	410,000
2,400	1,900	4,560,000	1,130	3,650	4,125,000
3,500	1,840	6,440,000	1,730	3,450	5,970,000
800	1,260	1,008,000	275	3,520	968,000
200	2,580	516,000	122	4,000	488,000

Chromatographie sur Décalso

Les hormones éluées du kaolin avaient des activités très voisines, 3,500 à 4,000 UI/mg cependant, l'électrophorèse libre montrait qu'elles étaient encore très impures (Fig. 1a).

Une chromatographie d'adsorption sur colonne de Décalso devait permettre de doubler cette activité. Le Décalso est un silicate synthétique de sodium et d'aluminium. Tamisé entre 80 et 100 mesh, il avait subi préalablement un cycle comportant un traitement par la soude normale, un lavage à l'eau, un traitement par l'acide acétique 0.25 *N*, un nouveau lavage à l'eau. Le Décalso était alors mis en colonne et lavé par le tampon utilisé au cours de l'adsorption, jusqu'à ce que le pH de l'effluent soit identique à celui du tampon. Une étude systématique avait permis de déterminer le pH et le tampon les plus favorables à la purification et au rendement de l'opération: c'était le tampon acétate 0.05 *M* de pH 5.4.

La capacité d'adsorption du Décalso est évidemment fonction du degré de pureté de l'hormone utilisée. Dans le cas présent, l'hormone titrant 3,000 à 4,000 UI/mg, la capacité du Décalso est d'environ 12,500 UI/g: ainsi, 80 g de Décalso étaient suffisants pour traiter 1,000,000 d'Unités, soient 250 à 300 mg du produit hormonal élué du kaolin. Cette quantité correspondait à des colonnes de 20 × 3 cm. La section importante des colonnes utilisées, $7\ cm^2$ permettait un écoulement plus rapide: toutes les chromatographies furent terminées en 8 h, adsorption et élution comprises. Le contact entre le Décalso et l'hormone doit être bref, afin d'éviter des dénaturations ou des adsorptions irréversibles.

La concentration en protéine des fractions collectées à la sortie de la colonne était suivie par un dosage spectrophotométrique à $278\ m\mu$. Dès que l'absorption dans l'u.v. redevenait nulle, l'élution était commencée: la fraction active devait être déplacée à un pH supérieur au pH d'adsorption. Pour réaliser cette élution dans un faible volume sans monter à un pH trop alcalin nuisible à l'hormone, il était nécessaire d'utiliser une solution à force ionique élevée, qui neutralisait rapidement le Décalso. Après divers essais, une élution rapide et quantitative fut obtenue par une concentration d'acétate d'ammonium de 10 % dans l'éthanol à 40 %; l'éthanol permettait de diminuer les quantités de protéines inactives éluées avec l'hormone.

La Fig. 2 est le schéma d'une chromatographie d'adsorption sur Décalso. Elle nous montre qu'une faible proportion des protéines n'était pas adsorbée et que la purification était surtout obtenue par élution fractionnée, un pourcentage important de protéines inactives n'étant élué que par des solutions très alcalines.

Cette opération permettait d'obtenir, par précipitation alcoolique des fractions

Schéma de la préparation de la Gonadatropine Choriale Humaine.

27 l d'urine titrant 23,000 UI/litre (621,000 UI)

Adsorbat benzoïque: 1950 mg (72.5 mg/l)

Extraction par le tampon acétate pH 4.5, $M/15$

Résidu: 1,085 mg

Extrait précipité par 5 volumes d'acétone
P = 785 mg 700 UI/mg, 549,000 UI
28 mg/l, 19,600 UI/l

Extraction par éthanol à 50 % dans
tampon acétate pH 4.5.+ CaCl, 0.015 M

Résidu: 390 mg

Extrait précipité par 5 volumes d'éthanol
312 mg 1,670 UI/mg, 521,000 UI
11.5 mg/l, 19,200 UI/l

Repris à pH 3
Précipité par 60 % d'éthanol

Surnageant précipité
à 85 % d'éthanol
85 mg

Précipité à 60 % d'éthanol
200 mg 2,580 UI/mg, 516,000 UI
7.4 mg/l, 19,100 UI/l

Adsorption sur kaolin

Surnageant du kaolin

Eluat kaolin précipité par 5 volumes d'acétone
122 mg 4,000 UI/mg, 488,000 UI
4.5 mg/l, 18,000 UI/l

Adsorption sur Décalso

Effluent

Eluat par acétate d'ammonium à 10 % dans éthanol
à 40 % – précipité par 5 volumes d'éthanol
69 mg 7,800 UI/mg, 538,000 UI
2.5 mg/l, 19,500 UI/l

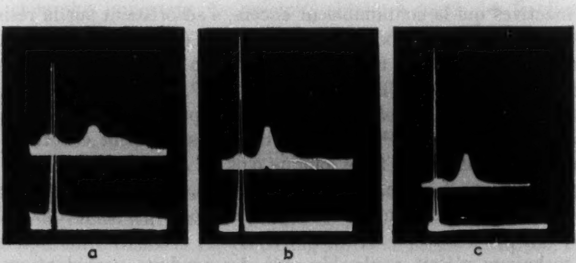

Fig. 1. Electrophorèses libres de préparations hormonales correspondant à différents stades de pureté. pH 8.6, 7,200 sec, 9.2 V/cm, μ = 0.1. a, 3,800 UI/mg; b, 7,000 UI/mg; c, 12,000 UI/mg.

éluées, une hormone titrant 7,000 à 8,000 UI/mg. Le rendement était proche de l'unité.

Grâce à des procédés originaux par leur utilisation présente, la méthode de préparation (schématisée dans le diagramme ci-contre) aboutissait à une hormone d'un titre ($9,200 > 7,500 > 5,700$, $P = 0.05$) supérieur au titre des hormones les plus actives préparées jusqu'alors. Cependant, cette hormone n'était pas encore homogène à l'électrophorèse (Fig. 1b).

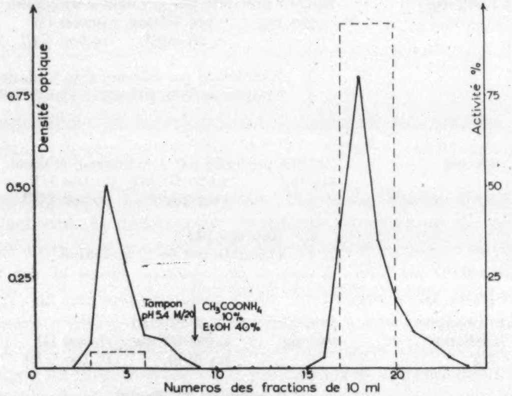

Fig. 2. Chromatographie sur Décalso d'une préparation titrant 3,800 UI/mg. ———, densité optique à 278 mμ; – – – –, rendement en % de l'activité initiale.

Obtention de l'hormone pure

Deux procédés ont permis d'obtenir la purification nécessaire.

Chromatographie sur Dowex 2: La technique utilisée est celle préconisée par BOMAN ET WESTLUND[11], pour la séparation de divers enzymes. Après traitement par l'acide chlorhydrique normal, la résine était tamponnée par un tampon "Tris" de pH 7.3 (voir réf. 12), et mise en colonne; une colonne de 20 × 2 cm suffisait pour un million d'Unités (40 g de résine). L'hormone, dissoute dans le tampon "Tris" 0.04 M, n'était pas adsorbée et se retrouvait directement dans l'effluent; seules les impuretés inactives qui la contaminaient encore, s'adsorbaient sur la résine et pouvaient être éluées par des tampons de molarités plus élevées. Le précipité alcoolique obtenu à partir de cet effluent, titrait 11,000 à 12,000 UI/mg. Le rendement de cette chromatographie était de 90 % environ.

Electrophorèse de zone sur colonne d'amidon: L'appareillage et la technique utilisés ont été décrits précédemment[13,14]. Les électrophorèses étaient effectuées à pH 8.6 en tampon véronal sodique de force ionique 0.05, à 4°, sous une tension de 400 V, avec un courant de 10 à 12 mA, durant 45 à 48 h. La concentration en protéine des fractions éluées des segments d'amidon, était dosée par la méthode de LOWRY *et al.*[15].

Cette technique n'était applicable qu'à des produits possédant une activité spécifique supérieure à 3,500 UI/mg, dans lesquelles la frontière hormonale était bien définie. En effet, dans des préparations moins pures, l'activité se répartissait sur une zone trop étalée au cours de l'électrophorèse.

La Fig. 3 représente la courbe de concentration protéique en fonction du nombre des segments d'amidon, après une électrophorèse effectuée sur un produit titrant 3,800 UI/mg. Les segments 12 à 19 correspondent aux impuretés inactives, encore présentes en majorité dans un tel produit; l'activité hormonale se trouve concentrée sur un seul pic, le plus lent (la mobilité électrophorétique de l'hormone est faible à pH 8.6: $-2.5 \cdot 10^{-5}$): le précipité obtenu à partir des fractions 7 à 11, pesait 70 mg et titrait 10,800 UI/mg, pour 200 mg de la substance initiale.

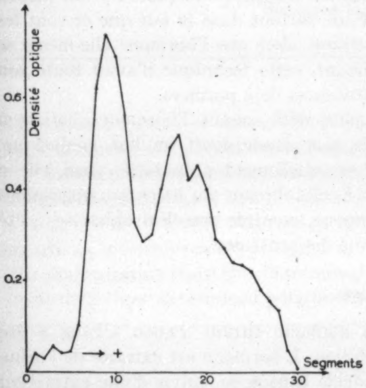

Fig. 3. Electrophorèse de zone sur amidon d'une préparation titrant 3,800 UI/mg.

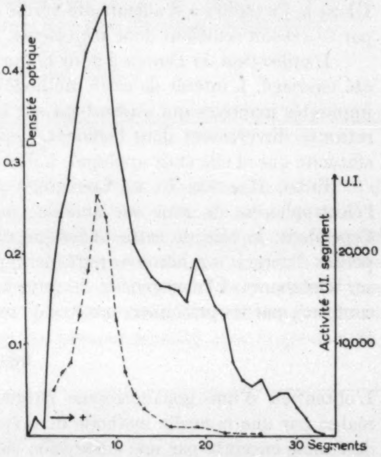

Fig. 4. Electrophorèse de zone sur amidon d'une préparation titrant 7,000 UI/mg. ●—●, concentration en protéine; ×---×, activité biologique.

Dans une hormone titrant 7,000 UI/mg, on ne trouve plus que 30 à 35 % d'impuretés; sur la Fig. 4 sont tracées les courbes de concentration en protéine et d'activité biologique des fractions électrophorétiques: la courbe d'activité coïncide avec le pic le plus lent et le plus important. Le précipité obtenu à partir des fractions 9 à 12 titrait 11,200 UI/mg, alors que pour les fractions 15 à 26, le précipité était totalement inactif.

Les hormones obtenues par ces deux procédés de purification étaient homogènes à l'électrophorèse (Fig. 1c).

DISCUSSION

La méthode de préparation décrite permet d'obtenir à partir de l'urine de femme enceinte, avec un rendement final de l'ordre de 70 %, une hormone dont l'activité spécifique se situe entre 10,000 et 12,000 UI/mg ($8,700 < 11,500 < 14,500$, $P = 0.05$), activité largement supérieure à celle des produits les plus purs déjà décrits dans la littérature? En effet, la seule gonadotropine choriale qui soit caractérisée sur le plan physico-chimique, a été préparée par GURIN *et al.*[2], mais son activité, exprimée en "unités animales" ne dépassait pas 6,000 m.e.d., et son estimation en Unités Internationales est délicate.

Plus récemment, CLAESSON *et al.*[3] avaient préparé une hormone plus active, titrant 6,000 à 8,000 UI/mg mais ils déclaraient extraire 20 mg de cette hormone par litre d'urine, soient 120,000 à 150,000 UI. Or, LORAINE[16] a montré que le maximum d'excrétion de la gonadotropine dans l'urine de femme enceinte est d'environ 50,000 UI/24 h. Ce chiffre a d'ailleurs été vérifié au cours de ce travail. Les chiffres avancés par CLAESSON semblent donc trop élevés.

L'utilisation du Dowex 2 pour la purification des gonadotropines n'avait jamais été envisagé. L'intérêt de cette méthode réside surtout dans le fait que ce sont les impuretés inactives qui s'adsorbent sur la résine, alors que l'hormone elle-même se retrouve directement dans l'effluent. Cependant, cette technique n'avait toute son efficacité que si elle était appliquée à des substances dejà purifiées.

Enfin, RAACKE, LI ET LOSTROH[17] avaient déjà soumis l'hormone choriale à l'électrophorèse de zone sur amidon; mais, leur étude avait un but analytique. Cependant, le rôle de cette technique est essentiellement préparatif: ainsi, elle a permis d'obtenir une hormone pure identique à celle obtenue par les chromatographies sur adsorbants. L'homogénéité de cette hormone, montrée par électrophorèse, a été confirmée par les principaux critères de pureté des protéines[5].

RÉSUMÉ

L'obtention d'une gonadotropine choriale humaine titrant 12,000 UI/mg a été réalisé par une nouvelle méthode de préparation. L'hormone est extraite de l'urine de femme enceinte par une adsorption sur acide benzoïque, suivie d'une extraction aqueuse à pH 4.5. Le produit brut ainsi obtenu subit alors une extraction hydro-alcoolique, par l'éthanol à 50 %, en présence de calcium, et l'hormone est précipitée à son pHi. Une adsorption sur kaolin, suivie d'une chromatographie d'adsorption sur Décalso, permet d'obtenir une substance titrant 7,000 UI/mg. Une dernière purification par chromatographie sur Dowex 2 ou par électrophorèse de zone sur amidon conduit à la Gonadotropine titrant 10,000 à 12,000 UI/mg et répondant aux principaux critères de pureté.

BIBLIOGRAPHIE

[1] P. A. KATZMAN ET E. A. DOISY, *J. Biol. Chem.*, 98 (1932) 739.
[2] S. GURIN, C. BACHMAN ET D. W. WILSON, *J. Biol. Chem.*, 128 (1939) 525.
[3] L. CLAESSON, B. HOGBERG, TH. ROSENBERG ET A. WESTERMAN, *Acta Endocrinol.*, 1 (1948) 1.
[4] P. A. KATZMAN, M. GODFRID, C. K. CAIN ET E. A. DOISY, *J. Biol. Chem.*, 148 (1943) 501.
[5] R. GOT ET R. BOURRILLON, *Bull. soc. chim. biol.*, 42 (1960) 31.
[6] E. DICZFALUSY, *Acta Endocrinol.*, 17 (1954) 58.
[7] J. S. L. BROWNE ET E. H. VENNING, *Lancet*, 2 (1936) 1507.
[8] H. M. EVANS, C. L. KOHLS ET D. H. WONDER, *J. Am. Med. Assoc.*, 108 (1937) 287.
[9] K. MEYER, A. LINDER, E. A. DAVIDSON ET B. WEISSMAN, *J. Biol. Chem.*, 205 (1953) 611.
[10] K. MEYER, E. A. DAVIDSON, A. LINDER ET P. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506.
[11] H. G. BOMAN ET L. E. WESTLUND, *Arch. Biochem. Biophys.*, 64 (1956) 217.
[12] G. GOMORI, *Proc. Soc. Exptl. Biol. Med.*, 62 (1946) 33.
[13] R. BOURRILLON ET R. GOT, *Bull. soc. chim. biol.*, 37 (1955) 915.
[14] R. BOURRILLON ET R. GOT, *Bull. soc. chim. biol.*, 41 (1959) 643.
[15] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR ET R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[16] J. A. LORAINE, *J. Endocrinol.*, 6 (1950) 319.
[17] I. D. RAACKE, C. H. LI ET A. LOSTROH, *Acta Endocrinol.*, 17 (1954) 366.

INFRARED SPECTRA OF CARBOHYDRATES IN WATER AND A NEW MEASURE OF MUTAROTATION*

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SUMMARY

The infrared spectra of various carbohydrates in water have been recorded and found to be characteristic of each compound.

Infrared techniques have been used to determine mutarotation constants of α-D-glucose, β-D-glucose and β-D-mannose. Absorbance values at a constant characteristic wavelength were observed as they changed with time; and were applied in an equation previously used in polarimetric determinations of the mutarotation constants. The mutarotation constants determined by the infrared method were in good agreement with those determined polarimetrically.

INTRODUCTION

In the past thirty years, scattered reports have appeared in the literature concerning infrared investigations in which solutions of certain carbohydrates were studied. In none of these reports, however, was any indication given that the spectrum of a sugar might change in solution with time owing to changes in the configuration of the sugar molecule.

An investigation was therefore undertaken to obtain the infrared spectra of various carbohydrates in water and to determine whether any configurational changes could be detected by infrared spectrophotometry. Although in recent years many excellent studies have been made of carbohydrates in the solid state, it should be kept in mind that water solutions can give information which is more characteristic of biochemical systems.

MATERIAL AND METHODS

The experimental techniques involved were essentially those previously published from this laboratory in other studies of biochemical substances, such as compounds of the citric acid cycle[1] and amino acids and related compounds[2,3]. The instrument used was a Perkin-Elmer model 21 infrared spectrophotometer with a sodium chloride prism. The sample solution was contained in a barium fluoride cell of 0.025 mm thickness. In the reference beam a transmittance screen was used. This screen could be adjusted to allow varying amounts of light through from the source. It was based on a screen mentioned by POTTS AND WRIGHT[4]. The carbohydrate solutions observed were either 10 or 20 % (w/v) in water.

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* A preliminary report was presented before the Division of Biological Chemistry of the American Chemical Society, in Atlantic City, New Jersey, September, 1959, *Abstracts of Papers, 136th Meeting, 1959*, 26C.

The materials studied were α-D-glucose (National Bureau of Standards, U.S.A.); β-D-glucose (Mann Research Laboratories (P), m.p. 149–150°, mutarotation: + 19.6 to 51.8° ($c = 10$); β-D-mannose (Mann, M.A., —16.0 to +14.3°); D-galactose (Pfanstiehl, anhydrous, C.P.); D-ribose (Nutritional Biochemicals Corp.); sucrose (J. T. Baker, analyzed); L-arabinose (Pfanstiehl, C.P.); D-levulose (Pfanstiehl, C.P.); glucose-1-phosphate, dipotassium salt (Schwarz Laboratories); and maltose hydrate (Pfanstiehl, C.P.).

RESULTS

The spectral change of α-D-glucose in water

Fig. 1 shows the spectrum of redistilled water in a 0.025 mm BaF_2 cell. The reference beam had only the transmittance screen. It can be seen that water has a transparent region from about 6.3 μ to 11 μ, which is the range used for these studies.

The observations from a set of experiments with α-D-glucose will now be described. It was considered possible at the outset that a band in the spectrum of α-D-glucose in water might be found which would change its intensity with time and thereby show up a structural change. Fig. 2 shows that a 20 % solution of α-D-glucose has such a band at 8.75 μ and that the transmittance of this band changes with time. Other bands also exhibit changes in their intensities with time, but the changes are smaller.

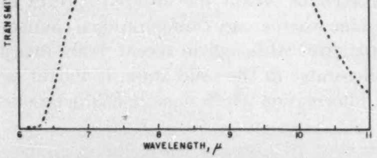


Fig. 1. Spectrum of water. Barium fluoride cell thickness 0.025 mm.

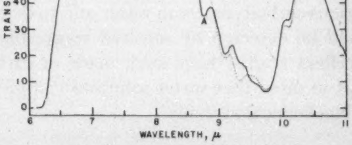


Fig. 2. —, the spectrum of α-D-glucose (20 % w/v) in water at time of dissolving; – – –, at end of mutarotation.

The recording of the curve (solid line) was begun at 6.10 μ, 2.5 min after water had been added to the sugar. Point A represents the intensity of the band at 8.75 μ, 4.3 min after water had been added. Point B (dotted line) represents the intensity of the band at 8.75 μ at the end of the run, 169 min after dissolution.

It was reasoned that if the change in intensity of the band at 8.75 μ is a measure of the change from α-D-glucose to the well-known equilibrium mixture, then the same band would show a reverse change in percent transmittance if a sample of β-D-glucose were dissolved in water.

The spectral change of β-D-glucose in water

Fig. 3 demonstrates the anticipated reverse effect at 8.75 μ. Point A lies on the curve (solid line) representing the run at the beginning of the mutarotatin of β-D-glucose. Point B lies on the curve (dotted line) representing the equilibrium mixture at the end of the run. The change in percent transmittance here is downward from A to B.

Kinetics of mutarotation

Further corroboration that changes in the band intensity at 8.75 μ are indicative of mutarotation was found when an equilibrium mixture containing 36.4 % α-D-glucose and 63.6 % β-D-glucose was dissolved in water[5] and observed in the spectrophotometer. Fig. 4 shows that the percent transmittance of the 8.75 μ band remained at a constant value for the whole duration of the experiment (triangles). In this figure are plotted the relative changes in percent transmittance for the α-form, the β-form, and the equilibrium mixture, *i.e.*, in each case the difference between the percent transmittance at 8.75 μ and the percent transmittance at the highest part of the curve (8.50 μ) was plotted on the ordinate as Δ percent *T*, *versus* time in minutes on the abscissa.

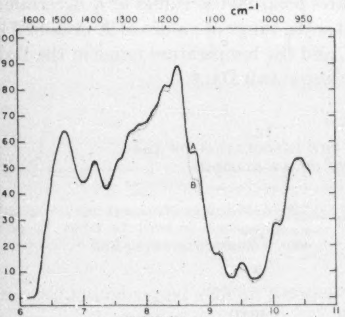


Fig. 3. —, the spectrum of β-D-glucose (20 % w/v) in water at time of dissolving; – – –, at end of mutarotation.

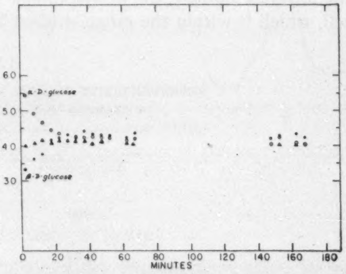


Fig. 4. ○, the relative changes in percent transmittance of the band at 8.75 μ for α-D-glucose; ●, β-D-glucose. ▲, for an equilibrium mixture. All in 20 % solutions.

It should be noted that the intensity changes of the α-D-glucose and of the β-D-glucose go in opposite directions and are essentially parallel to the effects seen in polarimetry.

The kinetics of such changes were calculated from absorbance values (as discussed below) for 10 % solutions observed in similar fashion. The reason for the use of 10 % solutions to calculate kinetic values was that previous workers[6,7], performing polarimetric measurements, had used solutions of 10 % or less; and consequently their values could be used for comparison with the results obtained in the present study. The range of K values (mutarotation constants) are in good agreement with those found by HUDSON AND DALE[6] by polarimetric methods.

The formulation of the first-order constant for the change, $\alpha \underset{k_2}{\overset{k_1}{\leftrightarrows}} \beta$, was used for glucose in the following way:

$$K = k_1 + k_2 = \frac{1}{t} \log \frac{A_0 - A_\infty}{A_t - A_\infty}$$

where, t is the time in minutes after addition of water; A_0 is the initial absorbance of the band at 8.75 μ; A_∞ is the final absorbance of the band at 8.75 μ; and A_t is the absorbance at time t of the same band, all values being read from a continuous plot at constant wavelength of absorbance *versus* time. The equation given above is an adaptation of the one given by HUDSON AND DALE[6], where absorbance values have been substituted for optical rotation values. The speed of recording was 12.5 min/μ for the runs at constant wavelength.

Table I presents six representative experimental points of A_t taken from a group of over 50 experimental points from a plot of absorbance *versus* time at constant wavelength (8.75 μ). The points given in the table for A_t are chosen randomly, but with the intent of showing the progressive change in A_t with time and the method of calculation. The data presented in the table show an upward drift in the K value with time, which is attributable to the range of temperature to which the solutions were exposed in the BaF_2 cell. However, the range of K values agrees well with the range of values determined by HUDSON AND DALE[6] for α-D-glucose and β-D-glucose, as seen in Table II. The second column indicates polarimetric values of K determined at 25°, 30° and 40°. The third column indicates the range of values of K obtained by the infrared method, the average value of K, and the temperature range in the BaF_2 cell, which is within the range studied by HUDSON AND DALE.

TABLE I

REPRESENTATIVE VALUES FROM THE CALCULATION OF THE MUTAROTATION CONSTANT OF α-D-GLUCOSE

$$K = \frac{1}{t} \log \frac{A_0 - A_\infty}{A_t - A_\infty}$$

A_0 = 0.347
A_∞ = 0.204
t = *minutes after addition of water*

t (min)	A_t	K
3.25	0.327	0.0199
8.25	0.301	0.0204
20.75	0.257	0.0207
32.00	0.234	0.0214
47.00	0.213	0.0262
62.00	0.208	0.0260

The mutarotation constants obtained for a 10 % solution of β-D-mannose under the same conditions (27–35°) ranged from 0.042 to 0.080, which also is in satisfactory agreement with HUDSON AND SAWYER's[7] reported values of 0.049 and 0.106 at 30° and 40°, respectively. Fig. 5 shows the changes undergone by β-D-mannose. The solid line represents the sugar shortly after dissolving. It can be seen that a band at 8.60 μ

TABLE II

A COMPARISON OF THE VALUES OF THE MUTAROTATION CONSTANT, *K*, OBTAINED BY HUDSON AND DALE[6] (POLARIMETRICALLY) WITH THE VALUES OBTAINED IN THE PRESENT STUDY (INFRARED)

Sugar	*K*	
	By polarimetry	*By infrared*
α-D-glucose	0.0105 at 25°	Temperature range, 27–35°
	0.0175 at 30°	0.0240 average
	0.0437 at 40°	(values range from 0.0186 to 0.0329)
β-D-glucose	0.01057 at 25°	Temperature range, 27–35°
	0.0168 at 30°	0.0200 average
	0.0395 at 40°	(values range from 0.015 to 0.0323)

has already begun to develop. The dotted line represents the sugar at the completion of mutarotation and indicates that the transmittance at 8.60 μ has decreased with time. Here, again, the mutarotation constant was calculated from absorbance readings at constant wavelength (8.60 μ), just as was done for glucose.

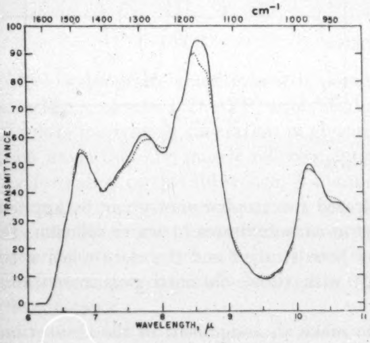


Fig. 5. —, the spectrum of β-D-mannose (20 % w/v) in water at time of dissolving; – – –, at end of mutarotation.

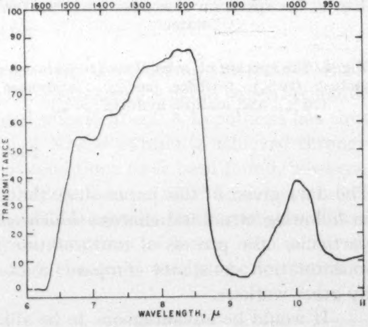


Fig. 6. The spectrum of glucose-1-phosphate, dipotassium salt (20 % w/v) in water.

Observations on sugars with no potential keto or aldehyde group

Fig. 6 shows the spectrum of glucose-1-phosphate (dipotassium salt). In this compound no mutarotation can take place because the hydroxyl group on carbon 1 has been esterified. No change in spectrum with time for this compound was observed. It is interesting, therefore, in this connection, that no absorption band is displayed at 8.75 μ, the band which undergoes the change in α-D-glucose and β-D-glucose.

Fig. 7 shows the spectrum of sucrose, which also cannot mutarotate. Again no change in spectrum with time was observed and there is no absorption band at 8.75 μ.

Spectra of some additional carbohydrates

Fig. 8 shows spectra of D-levulose (20 %), D-galactose (10 %), D-ribose (20 %), L-arabinose (20 %) and maltose hydrate (20 %) without regard for any possible mutarotation changes. The transmittance scales have been displaced, in order to spread out the five spectra. It can be seen that in water solutions, carbohydrates show characteristic spectra just as they do in the solid state. Each spectrum shown in Fig. 8 is different from any of the others shown in this paper.

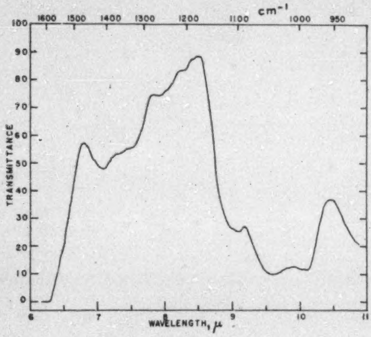
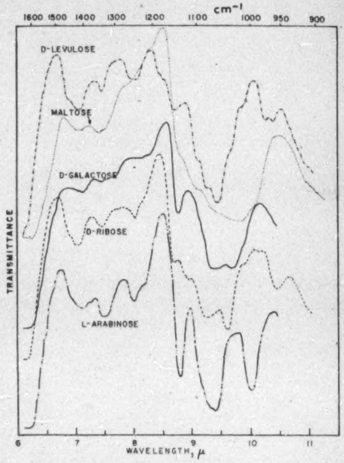


Fig. 7. The spectrum of sucrose (20% w/v) in water.

Fig. 8. The spectra of D-levulose (20%), D-galactose (10%), D-ribose (20%), L-arabinose (20%), and maltose hydrate (20%).



DISCUSSION

The data given in this paper show that infrared spectrophotometry can be applied in following structural changes which occur in carbohydrates in water solution. In particular, the process of mutarotation has been studied and the data relative to mutarotation constants compare favorably with those obtained polarimetrically by other workers.

It would be advantageous to be able to make an assignment of the absorption band at 8.75 μ (1143 cm^{-1}), but such an assignment cannot be made at this time. It has been stated[8] that, "beyond 960 cm^{-1}, C–O and C–C stretching, as well as C–H deformation and skeletal frequencies, make correlation between band positions and molecular structure difficult". Absorption due to the C–O–C antisymmetrical stretching has been observed[9, 10] in the region 1150–1080 cm^{-1} in the case of cyclic ethers, such as tetrahydrofuran and 1,4-dioxane, but there is still not enough information available to decide what the effects of the α- or β-configurations might be in this case.

ACKNOWLEDGEMENT

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REFERENCES

1 F. S. Parker, *Appl. Spectroscopy*, 12 (1958) 163.
2 F. S. Parker and D. M. Kirschenbaum, *Federation Proc.*, 18 (1959) 299.

[3] F. S. PARKER AND D. M. KIRSCHENBAUM, *J. Phys. Chem.*, 63 (1959) 1342.
[4] W. J. POTTS, JR. AND N. WRIGHT, *Anal. Chem.*, 28 (1956) 1255.
[5] For a discussion of the amounts of α- and β-D-glucose to be found at equilibrium, see F. DANIELS AND R. A. ALBERTY, *Physical Chemistry*, John Wiley and Sons, Inc., New York, 1955, p. 64.
[6] C. S. HUDSON AND J. K. DALE, *J. Am. Chem. Soc.*, 39 (1917) 320.
[7] C. S. HUDSON AND H. L. SAWYER, *J. Am. Chem. Soc.*, 39 (1917) 470.
[8] W. B. NEELY, *Advances in Carbohydrate Chemistry*, 12 (1957) 13.
[9] H. TSCHAMLER AND R. LEUTNER, *Monatsh. Chem.*, 83 (1952) 1502.
[10] S. A. BARKER, E. J. BOURNE AND D. H. WHIFFEN, in D. GLICK, *Methods of Biochemical Analysis*, Vol. III, Interscience Publishers, Inc., New York, 1956.

THE BIOSYNTHESIS OF A MUSCLE-RELAXING SUBSTANCE

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SUMMARY

Under appropriate conditions it is possible to show that, during the incubation of muscle microsomes with ATP, a soluble dialyzable substance is found which is capable of inhibiting tension generation in glycerinated muscle fibers. A hypothesis has thus been made that the muscle-relaxing activity of MARSH extract is achieved through the formation of this substance. A number of conditions have been found, however, where the microsomes were active, but no soluble substance could be demonstrated. The conditions most favorable for forming the soluble muscle-relaxing substance have been investigated and some preliminary characterization of it has been achieved. Evidence is presented which suggests that ATP is a substrate for the formation of the substance.

INTRODUCTION

Although the muscle-relaxing activity of extracts of muscle[1] is associated with the microsomal fraction of the extract[2–5], some doubt exists as to whether the microsomes per se produce relaxation. Both LORAND[6] and WEBER[7] have noted that the size of the microsomes would preclude their penetration into a glycerinated muscle fiber and consequently an essential area of the fiber would be beyond the action of the microsome. It thus becomes necessary to postulate that the microsomes produce relaxation by forming or releasing some readily diffusible substance. The observations that inhibition of tension[8] and inhibition of myofibrillar ATP-ase[9,10] is preceded by a latency which is abolished by preincubation suggests that such a substance is indeed formed, or released, or both. Attempts by NAGAI *et al.*[10] to demonstrate the presence of such a substance, however, met with failure.

Abbreviations used: ATP, adenosinetriphosphate; CP, creatine phosphate; CPT, creatine phosphoryltransferase; CTP, cytidine triphosphate; SMRS, soluble musele-relaxing system.

The present report shows that a dialyzable substance is formed or released by the interaction of ATP and either skeletal or cardiac muscle microsomes. This substance, like the Marsh extract, inhibits the tension induced by ATP in single glycerinated rabbit psoas muscle. PARKER AND GERGELY[11] have briefly reported a similar substance capable of inhibiting myofibrillar ATP-ase. Evidence is also presented which indicates that this substance is synthesized during the microsome-ATP incubation and not merely released.

METHODS

Muscle microsomes were prepared from rabbit skeletal muscle and dog cardiac muscle by differential centrifugation. The extract to be centrifuged was obtained by homogenizing chilled muscle in a Waring blendor for 40 sec with 3 volumes of a salt–sucrose solution (70 mM KCl, 2 mM $MgCl_2$, 20 mM Tris, 2.5 mM K-oxalate, 20 % sucrose). Brief periods of homogenization are essential when cardiac muscle is being used[12]. The homogenate was centrifuged for 25 min at 11,000 × g and the sediment discarded. The muscle microsomes were then obtained by centrifuging the supernatant for 1 h at 40,000 × g. The microsomes were resuspended in cold salt–sucrose solution so that 1 ml contained microsomal material from 2.5 g muscle. Resuspension using a Potter-Elvehjem homogenizer was necessary in order to obtain consistently active preparations.

Muscle-relaxing activity was assayed by its capacity to inhibit contraction in single glycerinated rabbit psoas fibers extracted for more than 90 days in 50 % glycerol at —18° (see ref. 13). Contraction was induced with a solution of 5 mM ATP, 2 mM $MgCl_2$, 70 mM KCl, 20 mM Tris, 2.5 mM K-oxalate, called the contraction solution. Experiments with glycerinated fibers were performed at pH 7 and room temperature, at a fiber length of 100 % rest length.

Protein was determined by the method of LOWRY *et al.*[14].

RESULTS

Fig. 1 shows the influence of incubation on the rate at which microsomes cause relaxation. Microsomes were added to contraction solution (see METHODS) either immediately or 48 min before relaxing activity of the incubation mixture (*i.e.* contraction

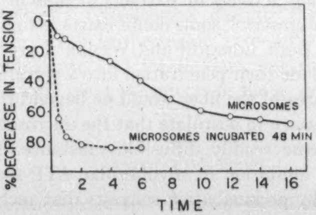

Fig. 1. Influence of preincubation of muscle microsomes on their relaxing activity. The curves depict the time course of fall in tension of a contracted fiber following the addition of microsomes or preincubated microsomes. The muscle fibers had been extracted for more than 6 months. ATP, 5 mM; Mg, 2 mM; μ = 0.16; room temperature; microsomes, 35 μg protein/ml contraction solution. For other details see text.

solution and microsomes) was to be tested. The intervals of time (0 and 48 min) and the concentration of granules used (35 μg protein/ml contraction solution) in this experiment were chosen arbitrarily to demonstrate most clearly the difference

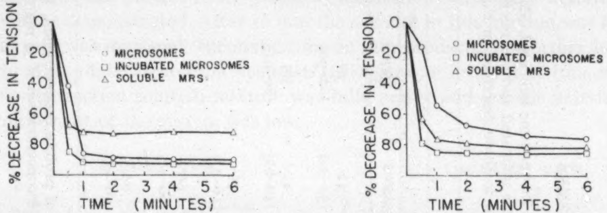

Fig. 2. Comparison of the tension-inhibiting activity of microsomes, incubated microsomes and SMRS. 2a shows such a comparison for microsomes prepared from rabbit skeletal muscle. The SMRS was prepared by centrifuging the incubation mixture for 1 h at 104,000 × *g*. 2b shows a similar experiment using microsomes prepared from dog myocardium. Incubation period was 30 min. Microsomes; 2a 380 μg protein/ml contraction solution. 2b, 1.1 mg protein/ml contraction solution. Conditions otherwise as in Fig. 1.

resulting from incubation. When a large amount of microsomes was employed the difference between incubated and non-incubated systems was much less striking (see Fig. 2a, 2b). The results shown in Fig. 1 confirm that a noticeable period exists before the microsomes become fully active and that this lag period disappears if the microsomes are preincubated. In most experiments the incubated and non-incubated microsomes eventually produced about the same degree of relaxation, suggesting that the material responsible for relaxation is being formed during the incubation.

A soluble muscle-relaxing system (SMRS)

If the incubation mixture described above, *i.e.* microsomes and contraction solution, was subjected to high speed centrifugation (104,000 × *g* for 1 h) to remove the microsomes, relaxing activity was still found in the supernatant. This supernatant will be referred to as the soluble muscle-relaxing system. Fig. 2a and 2b show the results of experiments conducted with rabbit skeletal muscle microsomes (Fig. 2a) or with dog cardiac microsomes (Fig. 2b), in which the activity of the SMRS is compared to that of incubated and non-incubated microsomal contraction solution mixture. Usually the SMRS did not produce as much relaxation as the non-sedimented incubation mixture. The relaxation produced by the SMRS was fully reversed by addition of calcium chloride.

Conditions favoring the formation of SMRS

It has never been possible to predict in advance the period of incubation or the amount of microsomes which would produce a maximally active SMRS. With each preparation of microsomes and even with the same preparation on different days the optimal incubation period and microsomal concentration varied. Table I is a summary of our experience to date. Both the average amount and the range of relaxing activity we observed after various incubation periods with various amounts of microsomes are shown in the table. Two points seem indicated: (a) within the range of concentrations at which microsomes are effective, the degree of inhibition of

TABLE 1

INFLUENCE OF INCUBATION TIME AND MICROSOMAL CONCENTRATION ON THE SMRS

MRF column shows the maximal depression (%) in tension obtained after the microsomes had been incubated for the time indicated. SMRS column shows the maximal depression (%) in tension obtained after the microsomes had been incubated for the time indicated and the microsomes had been spun down by high speed centrifugation. Conditions for determining inhibition of tension were identical to those described in Figs. 1 and 2.

*Microsome concn. (mg/ml)**	*Incubation period (min)*									
	6		*15*		*30*		*60*		*120*	
	MRF	*SMRS*	*MRF*	*SMRS*	*MRF*	*MSRS*	*MRF*	*SMRS*	*MRF*	*SMRS*
31			80 (76–84)	30 (11–43)	77 (75–80)	61	75 (70–80)	42 (32–52)	76 (62–90)	38 (35–40)
62					69 (65–80)	39 (19–69)	82 (78–84)	47 (40–62)	85 (80–91)	45 (26–74)
124	68 (64–73)	19 (8–25)	80 (77–82)	59 (23–69)	86 (84–89)	41 (17–73)	88 (85–91)	46 (16–77)		
248					79 (78–81)	63 (61–64)	81 (75–85)	66 (60–72)		
496					83 (79–85)	41 (32–50)	84 (82–86)	20 (0–33)		

* Since many different microsome preparations were used in this study, there was no absolute constancy of protein concentration. The amount of microsomes used, however, was always related to the amount of muscle from which they were prepared; thus a concentration of 31 mg/ml indicates that microsomes prepared from 31 mg of muscle were incubated per each ml of contraction solution.

tension is independent of the amount of microsomal material used; (b) the microsome–ATP mixture becomes fully active (*e.g.* 6 min) before the SMRS becomes fully active.

Fig. 3 shows in one series of experiments how the period of incubation influenced the formation of the SMRS. After 5 min of incubation only slight activity in the SMRS could be demonstrated. After 10 min the activity in this fraction was increased and by 20 min was maximal. Incubation for 60 min produced no further increment in activity. The 5-min incubation demonstrated again an interval of time when the microsome-contraction solution mixture was fully active and yet the activity of the soluble component of the system was low.

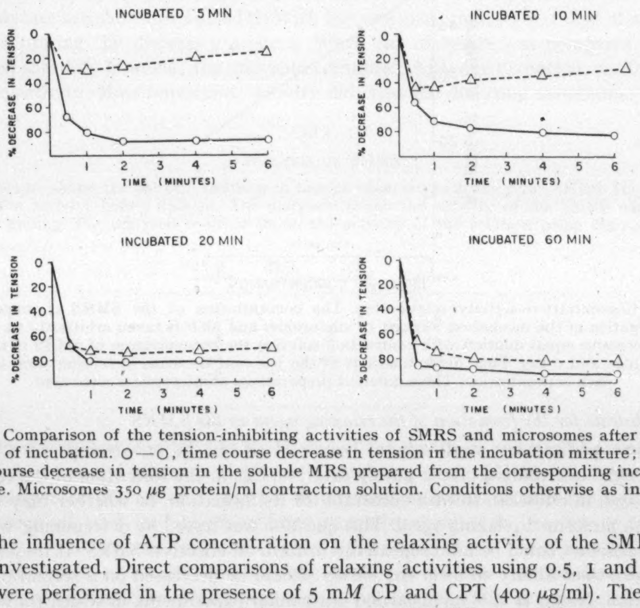
INCUBATED 5 MIN
INCUBATED 10 MIN
INCUBATED 20 MIN
INCUBATED 60 MIN

Fig. 3. Comparison of the tension-inhibiting activities of SMRS and microsomes after various periods of incubation. O—O, time course decrease in tension in the incubation mixture; △—△, time course decrease in tension in the soluble MRS prepared from the corresponding incubation mixture. Microsomes 350 μg protein/ml contraction solution. Conditions otherwise as in Fig. 1.

The influence of ATP concentration on the relaxing activity of the SMRS has been investigated. Direct comparisons of relaxing activities using 0.5, 1 and 5 m*M* ATP were performed in the presence of 5 m*M* CP and CPT (400 μg/ml). The transphosphorylating system was added to overcome ATP diffusion difficulties and the depression of ATP concentration by microsomal ATP-ase. Although the microsome preparations produced relaxation at 0.5 and 1 m*M* ATP no relaxing activity could be demonstrated in the SMRS. With one group of muscle fibers the cofactor described by Briggs *et al.*[15] was required to produce relaxation at both 0.5 and 1 m*M* ATP, but not at 5 m*M* ATP. The same microsome preparation when assayed on glycerinated fibers from another rabbit did not require cofactor. Since at 5 m*M* ATP the cofactor was not essential for the biosynthesis of active SMRS, it was not used in the preceding experiments. Such a use would have unnecessarily complicated the interpretation of the reaction leading to the formation of the active SMRS. The observation that the cofactor is required with some fibers and not with others, suggests that the fiber might be the site of action of the SMRS. Such a site has previously been suggested by Weber[7].

SMRS concentration–activity relationships

A total of 12 experiments were run to determine the effect of diluting SMRS on its activity. Fig. 4 shows that for each 50 % dilution there was approx. a 29% decrease in activity. The log of activity appears to be directly related to the concentration. The slope of this curve is very noticeably different from that relating the relaxing activity of muscle microsomes to their concentration[15]. Once an effective concentration of microsomes is reached, maximal relaxation is rapidly achieved.

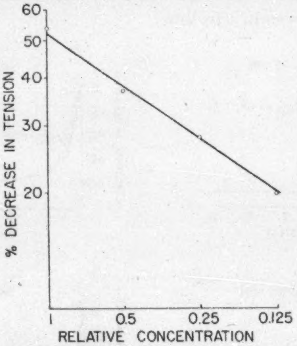

Fig. 4. Concentration–activity relationship. The concentration of the SMRS as prepared by centrifugation of the incubation mixture of microsomes and ATP is taken arbitrarily as 1. After each successive equal dilution with contraction solution the concentration of SMRS is taken to be 0.5, 0.25 and 0.125. Four determinations of the per cent decrease in tension were made at each concentration. Three different preparations of microsomes were used.

The substrate for the formation of the relaxing agent in the SMRS

Some preliminary studies have been carried out in an attempt to establish whether the microsomes play a purely catalytic role in the formation of the relaxing agent, and, in addition, furnish substrate for its formation, or whether they merely release a preformed relaxing agent. This question was tested by determining whether the microsomes could be used repeatedly to form an effective SMRS. If they furnish substrate, their ability to form the SMRS should be decreased on a second or third incubation. NAGAI *et al.*[10] have carried out similar experiments in which they tested the ATPase-inhibiting activity of microsomes after they had been repeatedly suspended in ATP. They found that after three such resuspensions the microsomes had

TABLE II

CAPACITY OF MUSCLE MICROSOMES TO FORM SMRS ON SUCCESSIVE REINCUBATIONS

The per cent decrease in tension reported is the maximal value. The microsomes were incubated in contraction solution for 30 min and centrifuged for 60 min at 104,000 × *g*. Conditions for measuring per cent decrease in tension were as described in Fig. 1.

Expt.	*Incubation mixture*	*SMRS 1*	*SMRS 2*	*SMRS 3*
	% decrease in tension	*% decrease in tension*	*% decrease in tension*	*% decrease in tension*
1	65	62	68	—
2	76	47	39	38
3	63	58	47	41

lost none of their capacity to inhibit ATP-ase. Table II shows that the capacity of the microsomes to form SMRS similarly was not significantly depressed when they were reused a second or third time. It was surprising, that considering the lability of the microsomes, there was not a greater loss in activity.

Preliminary characterization of the soluble muscle relaxing agent

Dialysis: The relaxing activity of the SMRS was found to be dialyzable. One volume of extraction solution was put into cellophane dialysis tubing and dialyzed against 10 volumes of SMRS. Dialysis was carried out for 16 h at 3°. The tension-inhibiting activity of the SMRS before dialysis, after dialysis, and the activity of the dialysate are shown in Table III. With but one exception, a great deal of activity was lost during the dialysis procedure. When the dialysate was compared to the dialyzed solution, however, the dialysate contained, with one exception, nearly comparable activity. Most important, activity did cross the dialyzing membrane.

TABLE III

DIALYSIS OF SMRS

Each column shows the per cent decrease in tension observed with the given SMRS. The control shows the activity before dialysis. The dialysate shows the activity of the SMRS within the dialysis tubing. The dialyzed solution shows the activity of the solution being dialyzed after dialysis.

Control	*Dialyzed solution*	*Dialysate*
46	15	18
45	28	11
40	46	40
38	27	21

Stability to heat and pH: Table IV shows the results of a series of experiments designed to test the influence of pH on the stability of the SMRS. When the SMRS was brought to pH 1.5 or 11 for 1 h at 0°, most of the tension-inhibiting activity was destroyed, and less deviation from neutrality resulted in less loss of activity. Heating at pH 7.0 was very deleterious; *e.g.* heating at 70° for 5 min resulted in about 50 % loss of activity and heating at 70° for 10 min resulted in more than 80 % loss of activity. The SMRS proved to be reasonably stable at room temperature. Activity was not measurably decreased in 2 h, the longest interval studied to date.

The relaxing activity in the SMRS is adsorbed by charcoal at pH 7.0. It is not

TABLE IV

STABILITY OF SMRS AT VARIOUS pH

The columns show the per cent decrease in tension produced by the SMRS before and after exposure to the indicated pH for 1 h at 0°.

Control	*pH 1.5*	*Control*	*pH 3.0*	*Control*	*pH 4.5*	*Control*	*pH 9*	*Control*	*pH 10*	*Control*	*pH 11*
46	7	42	37	70	29	42	24	78	18	60	8
49	11	61	30	43	42	61	40	50	13	49	18
				50	22			55	35		
				38	25						

as readily adsorbed, however, as ATP is. This characteristic has on occasion been successfully applied to the separation of ATP from the mixture.

DISCUSSION

The isolation by centrifugation of a dialyzable agent capable of inhibiting tension generation suggests the hypothesis that the function of the microsome in the muscle-relaxing system is not to cause relaxation directly, but to synthesize some agent which then produces relaxation. The time required to biosynthesize such an agent is thus very likely the cause of the delay observed before obtaining full inhibitory effects upon the addition of microsomes. The fact that the compound is dialyzable means that it could diffuse readily into the glycerinated muscle preparation, a point against the view that the microsome is the actual relaxing agent.

The inability to demonstrate active SMRS under all conditions in which the microsomes are effective, *e.g.* low ATP concentrations (0.5 and 1.0 m*M*) and brief periods of incubation at higher ATP concentrations (5 m*M*), raises some questions about this hypothesis. No certain explanation of the difficulty seems apparent at the moment. The inability to demonstrate the relaxing agent at low ATP concentration may in part be a reflection of the decreased relaxing ability of the microsomes at these concentrations of ATP[13] and the decreased amounts of relaxing factor that this represents. Even under apparently optimal conditions the success of preparing the relaxing agent was unpredictable. Obviously there are factors of still unknown significance playing a role in the system.

WEBER[7], in his discussion of the role of the microsomes in the relaxing mechanism, suggests two possibilities, either a relaxing agent is biosynthesized by the microsomes or the microsomes release a preformed agent to the myofibril. He suggests that perhaps ATP serves as a carrier in the system, since its presence is clearly requisite. This same explanation could be applied to the results obtained by preincubation and centrifugation. Certain observations seem to suggest that a preexisting relaxing agent in the microsome is unlikely. If such an agent did indeed exist, it should be possible by some means to release it from the microsome. Such techniques as freezing and thawing, sonication and treatment with organic solvents[16] have failed to produce any evidence of such a substance. Furthermore, the specificity of the relaxing system for ATP or CTP (see ref. 17), no other trinucleotide being effective, suggests a biochemical reaction rather than merely the release of a preexisting agent. The differing slopes relating tension-inhibiting activity to concentration of microsomes or SMRS suggest that the SMRS is of a different composition and hence must be formed during incubation.

The nature of the biosynthetic reaction leading to the formation of the relaxing agent remains to be elucidated. The experiments carried out so far add some information on this point. The following facts seem pertinent:

1. Only ATP and CTP of many nucleotides investigated are capable of producing relaxation[17].

2. The amount of microsomes used to form SMRS in the experiments reported above did not prove significantly related to the activity of the SMRS obtained. The capacity to form the SMRS was, however, related to ATP concentration. NAGAI[10] has presented contrary evidence, however, suggesting that a quantitative relationship

exists for the amount of microsomes needed to relax a given amount of myofibrils.

3. On successive incubations of microsomes with ATP the microsomes successively form the active agent. If the microsomes are donating a substance to this reaction no significant loss in this capacity was observed when the microsomes had been used for three successive incubations.

These data suggest that the ATP and, by analogy presumably CTP, function as one of the substrates for the formation of the active relaxing agent. Whether or not the microsomes also contribute substrate seems difficult to decide.

It is quite possible that ATP also functions as the energy source for the biosynthetic reaction. EBASHI[18] has attempted to discover, for example, whether the relaxing activity of microsomes could be dissociated from their ATP-ase activity. He found that, in all cases in which the ATP-ase had been destroyed, the relaxing function had been lost. He did find some situations in which the relaxing function was lost, but not the ATP-ase; he never found the converse to be true.

If ATP serves a substrate function in the formation of the relaxing agent, this has some important implications for the observation made by HASSELBACH AND WEBER[19] that relaxing factor shifts the region of over-optimal ATP concentrations to lower concentrations. This statement has had a certain mechanistic challenge. However, if low ATP concentration will not support the synthesis of the relaxing agent, but will support contraction[20], then a shift in overoptimal concentration need not be invoked to explain the results.

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REFERENCES

[1] B. B. MARSH, *Biochim. Biophys. Acta*, 9 (1952) 247.
[2] H. PORTZEHL, *Biochim. Biophys. Acta*, 26 (1957) 373.
[3] S. EBASHI, *Arch. Biochem. Biophys.*, 76 (1958) 410.
[4] J. R. BENDALL, *Nature* (1958) 1188.
[5] F. N. BRIGGS, J. GERGELY AND G. KALDOR, *Biochim. Biophys. Acta*, 34 (1959) 211.
[6] L. LORAND, *Conference on the Chemistry of Muscular Contraction*, Tokyo, 1957.
[7] H. H. WEBER, *Ann. N.Y. Acad. Sci.*, 81 (1959) 409.
[8] J. R. BENDALL, *Nature*, 170 (1952) 1058.
[9] J. R. BENDALL, *Nature*, 181 (1958) 1188.
[10] T. NAGAI, M. MAKINOSE AND W. HASSELBACH, cited by H. H. WEBER, in *Ann. N.Y. Acad. Sci.*, 81 (1959) 409.
[11] C. PARKER AND J. GERGELY, *Abstract 135th meeting American Chem. Soc.*, 1959.
[12] F. N. BRIGGS, *Federation Proc.*, 18 (1959) 372.
[13] F. N. BRIGGS AND H. PORTZEHL, *Biochim. Biophys. Acta*, 24 (1957) 482.
[14] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[15] F. N. BRIGGS, G. KALDOR AND J. GERGELY, *Biochim. Biophys. Acta*, 34 (1959) 211.
[16] J. GERGELY, G. KALDOR AND F. N. BRIGGS, *Biochim. Biophys. Acta*, 34 (1959) 218.
[17] W. HASSELBACH, *Biochim. Biophys. Acta*, 20 (1956) 355.
[18] S. EBASHI, *Arch. Biochem. Biophys.*, 76 (1958) 410.
[19] W. HASSELBACH AND H. H. WEBER, *Biochim. Biophys. Acta*, 11 (1953) 160.
[20] E. BOZLER AND J. T. PRINCE, *J. Gen. Physiol.*, 37 (1953) 63.

Short Communications

Effects of whole-body X-irradiation on the cholinesterase activity in liver and bone marrow of the guinea pig

In a previous communication[1] we reported on the early effects of whole-body X-irradiation on the cholinesterase activity in the blood of guinea pigs. A decrease of cholinesterase activity in plasma was found, but no significant alteration of acetylcholinesterase activity in the red corpuscles could be demonstrated. Since it may be supposed that the plasma cholinesterase changes could be connected with or possibly even preceded by similar changes in the liver, the chief site of synthesis of plasma proteins, and in organs engaged in haematopoiesis, we felt it worth-while to extend the investigation to include also the liver and bone marrow.

30 male guinea pigs with a mean body weight of 350 g were exposed to 400 R of X-irradiation (260 kV, 10 mA, filter 4 mm Al + 0.4 mm Cu, focus to target distance 40 cm, dose rate 123 R/min). 15 animals were killed at 24 h, and 15 animals at 48 h after irradiation; 15 animals served as nonirradiated controls.

Bone marrow was prepared from the hind legs after perfusion of the legs with normal saline. The marrow obtained was weighed and the activities of AChE and ChE were determined according to the method described by TAMMELIN *et al.*[2-4], using acetyl-β-methylcholine iodide and butyrylcholine iodide as substrates for the determination of AChE and ChE activity, respectively. 0.1 g of bone marrow was mixed with 7.5 ml of buffer solution[1], and 3 ml of the marrow-buffer solution + 0.3 ml of substrate solution were used for the determination of activity.

1 g of finely cut liver (perfused) was homogenized in 10 ml of buffer solution. The enzyme activities were determined in the same way as for bone marrow.

AChE and ChE activities in erythrocytes and plasma were determined according to the separated-blood method described earlier[1].

The results are presented in Fig. 1. The changes in the activities of the two cholinesterases in blood are in accordance with the results obtained in our previous study[1], although a lesser degree of statistical significance was obtained in the present material. In the liver no statistically significant changes in the activities of the cholinesterases have been found.

In the bone marrow the activity of both AChE and ChE is altered in the way that within 24 h the ChE activity increases up to about 150 % of that of the control animals and remains high during the following day. The AChE activity on the other hand, after two days is only about 70 % of that of the controls. The statistical significance of the values is indicated in the figure. In this connection it should be pointed out that, to our knowledge, there are no reports earlier in the literature about the occurrence of ChE in bone marrow[5].

Abbreviations: ChE, (pseudo)cholinesterase or butyrylcholinesterase; AChE, true acetylcholine-esterase.

Since no changes in the ChE activity in the liver could be demonstrated, the present investigation does not give any clue to a possible connection of a liver damage due to irradiation with the reduced ChE activity in plasma.

The alteration of the cholinesterase activities in the bone marrow indicates an influence of ionizing radiation on the metabolism of cholinesterases in this tissue.

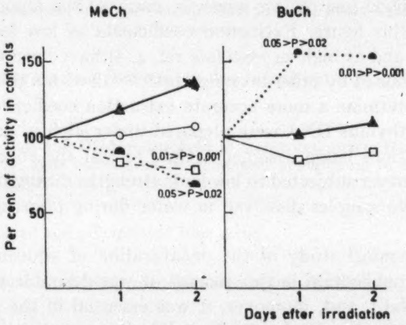

Fig. 1. ○, changes in cholinesterase activities in blood corpuscles; □, plasma; △, liver; ●, bone marrow of irradiated (400 R) guinea pigs. The values are expressed in per cent of normal activity values as determined by the hydrolysis of acetyl-β-methylcholine and butyrylcholine for AChE and ChE activity, respectively. Values of probability (P) are indicated for those points for which statistically significant differences from the normal material have been obtained.

It could be discussed, whether the cholinesterases are synthesized in the bone marrow, or if ChE is merely taken up in the marrow from the blood plasma, one mechanism or the other then being damaged by the ionizing radiation. The question of synthesis of the enzymes in the bone marrow implied by our results opens up a possibility to investigate from a new point of view the role of ChE in tissues. Concerning the possibility of an uptake of ChE in the bone marrow, the finding that the ChE activity in plasma diminishes after irradiation is of interest.

These results have been published as a contribution to the research on the effects of ionizing radiation on enzyme systems *in vivo* and to the discussion of the role of ChE for which enzyme neither a specific function nor a naturally occurring substrate has yet been demonstrated[6].

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[1] J. LUNDIN, C. J. CLEMEDSON AND A. NELSON, *Acta Radiol.*, 48 (1957) 52.
[2] L. E. TAMMELIN, *Scand. J. Clin. Lab. Invest.*, 5 (1953) 267.
[3] L. E. TAMMELIN AND H. LÖW, *Acta Chem. Scand.*, 5 (1951) 322.
[4] L. E. TAMMELIN AND B. STRINDBERG, *Acta Chem. Scand.*, 6 (1952) 1041.
[5] B. HOLMSTEDT, *Pharmacol. Revs.*, 11 (1959) 567.
[6] D. R. DAVIES, *J. Pharm. and Pharmacol.*, 4 (1954) 1.

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The u.v.-absorption of calf-thymus DNA

It is generally accepted that the extinction coefficient of DNA measured at 2590 Å is dependent on the degree of denaturation of a DNA solution. This is a consequence of the hyperchromicity displayed by native DNA on denaturation. The atomic extinction coefficient with respect to phosphorus of DNA prepared by mild methods is in the vicinity of 6600 (see ref. 1); however, many of the reported values lie in a broad range about this figure. Extinction coefficients as low as 5900 (see ref. 2), and 6000 (see ref. 3) and as high as 7500 (see ref. 4, 5) have been reported for native DNA. The values obtained by other investigators[6–10] fall within the region 6300–6600. In an attempt to determine a more accurate extinction coefficient for native DNA nine samples of calf-thymus DNA were prepared under slightly different conditions, the various modifications being designed mainly to test the effect of ionic history. Samples which were never subjected to low ionic strengths during preparation yielded an $\varepsilon(P)$ of 6540, while samples dissolved in water during preparation gave a value of 6660.

In a physico-chemical study of the denaturation of aqueous DNA solutions, to be submitted for publication in this journal, it was desirable to prepare samples of DNA free from NaCl, and, moreover, it was essential in the preparation to use aqueous solutions without added salt. To fulfil these conditions it was necessary to carry out the final precipitation with ethanol from a salt-free solution rather than from 1 *M* NaCl as commonly employed. The u.v.-absorption of various samples subjected to this treatment was compared with measurements made on DNA samples that had never been dissolved in water of low ionic strength. Table I lists the preparative histories of the nine samples used in this and later investigations.

The u.v.-measurements were made on solutions of DNA in 0.1 *M* NaCl prepared in one of two ways: (a) DNA was dissolved in water, at 4°, to give a concentration of $3 \cdot 10^{-3}$ *M* with respect to phosphorus and subsequently made 0.1 *M* with respect to NaCl by the addition of concentrated salt solution and then diluted to $5 \cdot 10^{-5}$ *M* DNA with 0.1 *M* NaCl; (b) DNA was dissolved directly in 0.1 *M* NaCl and later diluted with 0.1 *M* NaCl to $5 \cdot 10^{-5}$ *M* DNA. All $\varepsilon(P)$ values refer to the phosphorus concentration determined on each stock solution.

As no significant difference was noticed between methods (a) and (b) for the samples that had been dissolved in water of low ionic strength, these results were pooled and are shown in Table II, about equal numbers of both samples being used. The extinction coefficient of the samples dissolved in water during preparation was found to be 6660 ± 30. The results for the DNA samples never dissolved at low ionic strength during preparation are given in Table III. It can be seen that a small but significant difference between methods (a) and (b) was found. If the sample was dissolved in water before adjusting to 0.1 *M* NaCl a value of 6630 was obtained in agreement with the results expected from Table II; however, if the sample was dissolved directly in 0.1 *M* NaCl an average value of 6540 was obtained. This value is to be regarded as the extinction coefficient of native DNA as prepared by the detergent method[11] and is very close to the value of 6650 ± 50 recorded by CHARGAFF AND LIPSHITZ[12], although these measurements were made before the possibility of denaturation of DNA by exposure to solutions of low ionic strengths was fully

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Abbreviation: DNA, deoxyribonucleic acid.

TABLE I

PREPARATIVE HISTORIES OF NINE SAMPLES OF CALF-THYMUS DNA

Preparative history	*Dissolutions in water*
Preparation 1 Prepared by the detergent method[11] except that the final fibres were washed with ethanol only. Dried over P_2O_5 for 2 days *in vacuo*.	2
Preparation 2 As for 1, except that the final precipitation was carried out from aqueous solution. Dried over P_2O_5.	2
Preparation 3 As for 2, except that the DNA redissolved in water and precipitated from saline solution three times then redissolved in water and precipitated in the absence of salt. Dried over P_2O_5.	4
Preparation 4 Portion of 3 dissolved and precipitated from water.	5
Preparations 5 and 6 As for 1, except that 0.1 M NaCl used instead of water at all stages. Air dried.	0
Preparation 7 Prepared by the method of GULLAND *et al.*[13] and finally dissolved and precipitated from water.	2
Preparations 8 and 9 As for 1, except that 0.0014 M NaCl was used instead of water at all stages. Air dried.	0

TABLE II

ATOMIC EXTINCTION COEFFICIENTS MEASURED AT 2590 Å OF DNA SAMPLES DISSOLVED IN WATER DURING PREPARATION

Preparation	*Number of measurements*	*ε(P) Pooled results of methods (a) and (b)*	*Standard error*
1	14	6614	26
2	26	6646	23
3	18	6651	20
4	44	6705	12
7	25	6613	22
		Average 6657	

appreciated. It can be concluded that dissolution of native DNA in water at a concentration of $3 \cdot 10^{-3}\,M$ results in an irreversible increase in extinction coefficient of 1.4 %, which is compatible with a small amount of denaturation. Unfortunately this small amount of denaturation will complicate any study involving aqueous solutions of low ionic strength.

The possible denaturing effect of precipitation with ethanol, which might be present in all the results so far given, was checked as follows. The ε(P) was determined for a solution of preparation 6 before precipitation with ethanol after the first deproteinisation, and corrected for the residual detergent present. The value was found

TABLE III

ATOMIC EXTINCTION COEFFICIENTS MEASURED AT 2590 Å OF DNA SAMPLES NEVER DISSOLVED IN WATER OF LOW IONIC STRENGTH DURING PREPARATION

Preparation	$\varepsilon(P)$	
	Method (a)	*Method (b)*
5	6540	6570
6	6690	6530
8	6670	6560
9	6620	6490
Average	6630	6540

to lie between 6580 and 6630 and was not significantly different from the overall mean value.

A source of error in the measurement of extinction coefficients in the case of DNA is apparent on inspection of the water content of solid samples. For the preparations initially dried over P_2O_5 the water content displayed a general rise from 5–12 % up to 20–30 % on exposure to atmospheric humidity. In all the preparations studied, however, there was never any predictable change in water content except for the general rise noted above. Because of the variation in water content any absorptivity measurements which ultimately depend on the weighing out of DNA will be inviting error. This could come about, for instance, if the phosphorus content of a particular preparation was determined and at a later date the u.v.-measurements made on a different weighed DNA sample.

The Commonwealth Scientific and Industrial Research Organisation Division of Mathematical Statistics very kindly carried out the statistical analysis of the results given in this investigation.

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[1] E. CHARGAFF, *The Nucleic Acids*, Vol. 1, Academic Press, 1955, p. 336.
[2] K. S. KIRBY, *Biochem. J.*, 66 (1957) 495.
[3] P. D. LAWLEY, *Biochim. Biophys. Acta*, 21 (1956) 481.
[4] J. POUYET AND G. WEILL, *J. Polymer Sci.*, 23 (1957) 739.
[5] C. D. JARDETZKY, *J. Am. Chem. Soc.*, 80 (1958) 1125.
[6] L. F. CAVALIERI, M. ROSOFF AND B. H. ROSENBERG, *J. Am. Chem. Soc.*, 78 (1956) 5239.
[7] E. L. DUGGAN, V. L. STEVENS AND B. W. GRUNBAUM, *J. Am. Chem. Soc.*, 79 (1957) 4859.
[8] E. CHARGAFF, E. VISCHER, R. DONIGER, C. GREEN AND F. MISANI, *J. Biol. Chem.*, 177 (1949) 405.
[9] R. THOMAS, *Bull. soc. chim. biol.*, 35 (1953) 609.
[10] E. P. GEIDUSCHEK, *J. Polymer Sci.*, 31 (1958) 67.
[11] E. R. M. KAY, N. S. SIMMONS AND A. L. DOUNCE, *J. Am. Chem. Soc.*, 74 (1952) 1724.
[12] E. CHARGAFF AND R. LIPSHITZ, *J. Am. Chem. Soc.*, 75 (1953) 3658.
[13] J. M. GULLAND, D. O. JORDAN AND C. J. THRELFALL, *J. Chem. Soc.*, (1947) 1129.

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Photodynamic inactivation of transforming principle

Photodynamic action is the dye-sensitized autoxidation of a biological substrate accelerated by the action of visible light[1]. Using an aromatic amine as the substrate we have demonstrated that the dye-sensitized photo-oxidation in this case involves the reaction of oxygen with the dye molecules in the long-lived (triplet) excited state[2]. Many types of dyes, such as azo dyes or triphenylmethane dyes (in the free state) do not undergo transitions to the triplet state, and hence are not photosensitizers. It is of interest to determine whether those dyes, which are sensitizers for the synthetic substrate, are also effective for a biological substrate.

We have investigated the dye-sensitized photo-inactivation of transforming principle since it is a relatively pure substance which loses its biological activity when no physical changes in its structure are evident. DNA* was prepared from a rough strain of *Diplococcus pneumoniae* which was resistant to streptomycin[3].

Assays for the transforming activity of the DNA were carried out in a manner identical with that described by ROSENBERG *et al.*[4]. It was found that at DNA concentrations greater than 2 μg/ml the transforming activity was independent of concentration. All subsequent determinations were made at three different DNA concentrations greater than this value. The average percentage of cells transformed was about 5.6 %.

For the determination of the photodynamic inactivation of the transforming activity of DNA, part of a solution containing 23 μg DNA/ml was used as a control, and dye was added to the remainder to give a final dye concentration of $5 \cdot 10^{-6}$ *M*. Aliquots of these solutions were irradiated for varying lengths of time, using a 500-W tungsten-lamp projector with a u.v.-cut-off filter (Corning No. 3–74) to eliminate radiation below 400 mμ. The samples were illuminated at a distance of 15 cm from the front surface of the projector lens so as to eliminate heating effects. In each case a sample not containing dye was also illuminated, and served as a second control sample: in no case did those samples differ in transforming activity from samples which did not contain dye, and which had not been irradiated. All samples were then tested for transforming activity, each inoculum tube containing 2.3, 3.5, and 4.6 μg DNA/ml, with $5 \cdot 10^{-7}$, $7.5 \cdot 10^{-7}$ and 10^{-6} *M* dye, respectively. Where photodynamic inactivation occurred the remaining transforming activity was in all cases still independent of DNA concentration in the concentration range chosen. The transforming activities given in Table I represent the average of triplicate assays at the three different DNA concentrations and are in error by no more than 10 %.

In Fig. 1 it is seen that about half the original activity (5399 cells transformed per ml of suspension) is lost after 15-min irradiation in the presence of methylene blue. There is no photodynamic effect in the absence of dye, and the dye has no effect in the dark. In Table I are listed the results for 15-min irradiation with various dyes. The dyes were used in $5 \cdot 10^{-6}$ *M* concentration in the original solutions so that upon addition to the inoculum the viability of the cells was unimpaired. This was checked by performing total counts on inocula with and without dye present. Although

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Abbreviations: DNA, deoxyribonucleic acids.

* The preparations of DNA having transforming properties and its assay were carried out in the laboratory of Dr. D. L. HUTCHINSON at the Sloan Kettering Institute in cooperation with Dr. F. M. SIROTNAK, whose many helpful suggestions are hereby gratefully acknowledged.

none of the dyes are bactericidal at the concentrations employed, both Thioflavin TG and Proflavine decrease the transforming activity, even in the absence of light, and any photo-inactivation phenomena are thus masked. These observations may possibly be correlated with the known mutagenic activity of acriflavine, of which proflavine is the principal constituent. Our failure to demonstrate photodynamic inactivation with proflavine is in agreement with the observation that acriflavine failed to photosensitize the inactivation of the transforming principle of *Hemophilus influenzae*[5], although here no dark effect was observed.

TABLE I

Dye	*Control (no dye) Transforming activity**	*Activity and control*	
		Dye alone % of	*Dye and light*
Rose bengal	1212	100.5	59.8
Methylene blue	5368	95.6	44.0
Proflavine	1682	68.2	64.2
Neutral red	1682	110.1	40.6
Crystal violet	1682	101.4	72.1
Thioflavine TG	1682	71.0	63.2
Cresyl violet	1055	105.3	93.0
Methyl orange	1433	100.5	98.9
Alizarin red	1641	96.2	97.9
Riboflavin	9120	89.9	51.9

* "Transforming activity", the number of cells transformed per ml of suspension under the conditions of assay as outlined in the text.

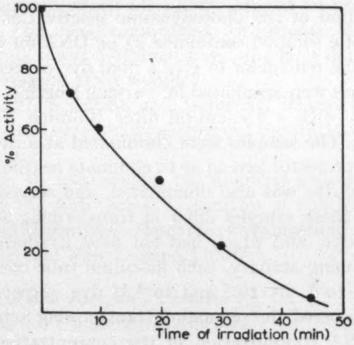

Fig. 1. Rate of photodynamic inactivation of Transforming Principle. $3.5 \cdot 10^{-6}$ *M* methylene blue was used as sensitizer. The oridinate indicates the remaining activity as a percentage of the original activity.

The anthroquinone dye alizarin red, the azo dye methyl orange, and the oxazine dye cresyl violet are without photodynamic effect on the transforming principle, as they are when *p*-toluenediamine is employed as a substrate. The triphenylmethane dye crystal violet acts as a sensitizer because it is bound to the nucleic acid, and, again in conformity with prior observations using the aromatic amine as substrate, it can act as a photosensitizer in the bound state. Rose Bengal (a xanthene dye), methylene blue (a thiazine dye), and riboflavine, are as efficient sensitizers for the inactivation of transforming principle as they are for the photosensitized oxidation of *p*-toluenediamine. Contrary to our previous report[2], we have since found that in the strict absence of oxygen, and using ethylenediaminetetraacetic acid as electron donor, neutral red (an azine dye) can be photoreduced. In the presence of no more than a trace of oxygen it can sensitize the photopolymerization of acrylamide. Our failure to control the oxygen level accounts for the difference between the present results and those obtained earlier. Spectrophotometric studies also show that neutral red is a sensitizer for the photodynamic oxidation of *p*-toluenediamine. In conformity with these findings the present study shows that neutral red is also able to act as sensitizer for the photodynamic inactivation of transforming principle.

Thus, in every case the ability of the dyes to photosensitize the autoxidation of the organic substrate is paralelled by their ability or inability to sensitize the inactivation of transforming principle.

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[1] H. F. BLUM, *Photodynamic Action and Diseases Caused by Light*, Reinhold Publishing Corporation, New York, 1941.
[2] G. OSTER, J. S. BELLIN, R. W. KIMBALL AND M. E. SCHRADER, *J. Am. Chem. Soc.*, 81 (1959) 5095.
[3] E. R. M. KAY, N. S. SIMMONS AND A. L. DOUNCE, *J. Am. Chem. Soc.*, 74 (1952) 1724.
[4] B. H. ROSENBERG, F. M. SIROTNAK AND L. F. CAVALIERI, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 14
[5] S. ZAMENHOF, G. LEIDY, E. HAHN AND H. E. ALEXANDER, *J. Bacteriol.*, 72 (1956) 1.

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The isolation of bacterial mutants defective in amino acid transport

Many bacterial strains accumulate amino acids from the surrounding medium[1-4]. The mechanism is obscure, but the phenomenon clear. SCHWARTZ, MAAS AND SIMON[5] have reported that mutants of *Escherichia coli* W with an impaired concentrating mechanism for arginine can be isolated by selection on canavanine-supplemented plates, and for glycine by selection on D-serine-supplemented plates.

We have successfully used another method for isolation, and have obtained three mutant types of *E. coli* W which are defective in the ability to accumulate either histidine, proline or glycine. The first two are new; the last one presumably similar to that isolated by SCHWARTZ *et al.*[5].

For simplicity, these mutants are referred to below as transport-negative (Tr^-). These mutants may be analogous to the permease mutants of the β-galactoside system[4,6], but we prefer the general term "transport" because the process is not understood.

The isolation procedure starts with the use of an auxotroph, *e.g.*, His^-, deficient in some step in histidine biosynthesis, but capable of accumulating histidine from the medium. The wild type (with respect to the transport character), hence $His^-Tr^+_{his}$, grows well on media or plates containing as little as 10 μg/ml L-histidine, although the final concentration of bacteria reached after growth, or the colony size on plates, is limited. The mutant, His$^-$Tr$^-_{his}$, will grow rapidly only when large amounts—greater than 250 μg/ml—are supplied. At high levels, the supplement presumably diffuses in at a sufficient rate so that the rate of growth is no longer limited by the size of the internal pool of required nutrient.

An overnight culture of a His$^-$Tr$^-_{his}$ auxotroph of *E. coli* W was irradiated with u.v. light to about 0.1 % survival. 5 ml of the irradiated culture were grown in 50 ml of minimal medium A (see ref. 7) supplemented with 1 mg/ml L-histidine. After overnight growth, the cells were centrifuged, washed in buffer, and diluted in 15 ml of A plus 10 μg/ml histidine. The culture was shaken at 37° for 1 h, penicillin added (final concn., 2000 units/ml), and the incubation continued. To follow the time course of killing, three serial samples, appropriately diluted, were spread on nutrient plates at approximately hourly intervals, according to the modified penicillin selection method of GORINI AND KAUFMAN[8].

At these same intervals, about 5 ml of the culture were passed through an HA-Millipore filter* (pore size 0.45 μ), and washed with buffer. The filter, with adherent bacteria, was pushed into a tube containing medium A plus 1000 μg/ml histidine. After growth overnight, the bacterial culture in the tube corresponding to the sample which showed considerable killing by penicillin, was treated further. The cells were centrifuged, washed, and treated exactly as the day before. After two cycles of selection in penicillin and regrowth, appropriate dilutions were spread on plates containing 1 mg/ml histidine, with subsequent replica plating[9] on 10 μg/ml plates. On 1 mg plates, His$^-$Tr$^+_{his}$ and His$^-$Tr$^-_{his}$ colonies were similar in size; on 10 μg plates His$^-$Tr$^+_{his}$ grew to small-sized colonies, but His$^-$Tr$^-_{his}$ colonies were invisible to the naked eye.

Similar procedures with minor modifications were used to isolate the other mutants. The glycine transport-negative mutants were found readily; after one cycle of penicillin treatment, more than 25 % of the survivors were Gly$^-$Tr$^-_{gly}$. This frequency is consistent with the large number of mutants in *E. coli* W resistant to D-serine, as described by DAVIS AND MAAS[10]. Selection methods using D-serine, which readily produce transport-negative mutants (Tr$^-_{gly}$) of *E. coli* W fail for *E. coli* B, which is not sensitive to this inhibitor. Gly$^-$Tr$^-_{gly}$ mutants of *E. coli* B, however, were easily isolated by the penicillin method. For isolation of His$^-$Tr$^-_{his}$ and Pro$^-$Tr$^-_{pro}$, two or three cycles of treatment with penicillin were necessary, and only a few were found among several hundred colonies examined.

A reconstruction experiment, using His$^-$Tr$^+_{his}$ S^s and His$^-$Tr$^-_{his}$ S^r (S^s = streptomycin sensitive; S^r = streptomycin resistant) showed that the selection technique did not produce significant death of the Tr$^-_{his}$ mutant during treatment with penicillin. All Tr$^-_{his}$ mutants isolated have shown measurable reversion rates to Tr$^+_{his}$. Reversion from Tr$^-_{his}$ to Tr$^+_{his}$ is independent of reversion from the auxotroph to prototroph state. When colonies on plates or slants containing 500 to 1000 μg/ml of supplement are tested after many days of storage at 5–10°, the proportion of Tr$^+_{his}$ and His$^+$ in an originally pure His$^-$Tr$^-_{his}$ colony rises. This may be due to depletion of supplement in the agar which surrounds the colony, resulting in a growth advantage for Tr$^+_{his}$ or

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* Millipore Filter Corporation, Bedford, Mass. (U.S.A.).

His$^+$ revertants. Colonies on slants need frequent retesting, and storage in frozen form with 5 to 10 % glycerol is preferred.

Studies of the rate of uptake of ^{14}C-labeled amino acids show that the defect is selective. Cultures were grown to the exponential phase in a medium containing 500 µg/ml of the required amino acid. Chloramphenicol was added (final concn. 200 µg/ml) and the cells ($5 \cdot 10^8$–10^9/ml) incubated for 30 min. The cells were then washed, resuspended in medium A + 50 µg/ml chloramphenicol and reincubated for an additional 30 min. The specified [^{14}C]amino acid was added, and incubation continued for 5 min. An aliquot was withdrawn, filtered through an HA-Millipore filter, and the radioactivity in the washed bacteria measured with a Nuclear-Chicago thin-window counter. The figures in Table I are corrected for the radioactivity which remains in the cellular residue after 5 min at 100°. This correction amounts to at most 5 % of the observed count for both Tr$^+$ and Tr$^-$ mutants.

The difference in histidine content of $\mathrm{Tr}^+_{\mathrm{his}}$ and $\mathrm{Tr}^-_{\mathrm{his}}$ mutants can be seen with 10 µg/ml [^{14}C]histidine in the medium (Table I). The disparity is accentuated when only 1 µg/ml is present, reaching a ratio for $\mathrm{Tr}^+_{\mathrm{his}}/\mathrm{Tr}^-_{\mathrm{his}}$ of about 35. The expected distribution of L-histidine due to passive diffusion along electrochemical gradients cannot be calculated with confidence because several physical parameters are unknown, *e.g.*, the intracellular water space of the bacterium and the difference in electrical potential between intracellular and extracellular fluids. The quantity of histidine in $\mathrm{Tr}^-_{\mathrm{his}}$, however, is close to that predicted by the assumption of passive distribution. The physical state of the amino acid accumulated by the $\mathrm{Tr}^+_{\mathrm{his}}$ mutants is open to conjecture. Preliminary experiments with intact[11] and lysed protoplasts of Gly$^-$ $\mathrm{Tr}^+_{\mathrm{gly}}$ and Gly$^-$$\mathrm{Tr}^-_{\mathrm{gly}}$ show that the cell fraction which includes cell membrane is partly responsible for the transport specificity.

TABLE I

ACCUMULATION OF RADIOACTIVE AMINO ACIDS BY TRANSPORT-POSITIVE AND TRANSPORT-NEGATIVE AUXOTROPHS IN *Escherichia coli* W.

Bacterial population about 10^9 cells/ml. Medium contained 50 µg/ml chloramphenicol. L-proline and L-histidine were randomly labeled. Glycine, DL-phenylalanine and DL-lysine were labeled in the C-2 position. Samples were taken 5 min after addition of radioactive amino acid. Only one amino acid was added to any tube, and experiments on each pair of mutants were run in parallel. Data assume complete equilibration of [^{12}C]- and [^{14}C]amino acids.

Mutant	*Concentration of amino acid in medium in µg/ml*	*(µg/10^{12} cells)*				
		Glycine	*Proline*	*Phenylalanine*	*Histidine*	*Lysine*
His$^-$$\mathrm{Tr}^+_{\mathrm{his}}$	10	140	160	135	600	400
His$^-$$\mathrm{Tr}^-_{\mathrm{his}}$	10	250	200	90	92	360
His$^-$$\mathrm{Tr}^+_{\mathrm{his}}$	1	—	—	—	540	—
His$^-$$\mathrm{Tr}^-_{\mathrm{his}}$	1	—	—	—	15	—
Gly$^-$$\mathrm{Tr}^+_{\mathrm{gly}}$	10	360	225	160	500	140
Gly$^-$$\mathrm{Tr}^-_{\mathrm{gly}}$	10	40	250	140	500	145
Gly$^-$$\mathrm{Tr}^+_{\mathrm{gly}}$	1	400	—	—	—	—
Gly$^-$$\mathrm{Tr}^-_{\mathrm{gly}}$	1	10	—	—	—	—
Pro$^-$$\mathrm{Tr}^+_{\mathrm{pro}}$	10	400	176	400	500	370
Pro$^-$$\mathrm{Tr}^-_{\mathrm{pro}}$	10	600	64	320	310	260
Pro$^-$$\mathrm{Tr}^+_{\mathrm{pro}}$	1	—	165	—	—	—
Pro$^-$$\mathrm{Tr}^-_{\mathrm{pro}}$	1	—	15	—	—	—

Growth curves in liquid media for His$^-$$\mathrm{Tr}^+_{\mathrm{his}}$ and His$^-$$\mathrm{Tr}^-_{\mathrm{his}}$ are identical when the histidine level is above 250 μg/ml. At 100 μg/ml, marked slowing of $\text{His}^-\text{Tr}^-_{\text{his}}$ growth appears. At 10 μg/ml the population size of $\text{His}^-\text{Tr}^-_{\text{his}}$ is stationary while $\text{His}^-\text{Tr}^+_{\text{his}}$ grows rapidly. Assay of the medium before and after incubation with $\text{His}^-\text{Tr}^-_{\text{his}}$ showed no destruction of histidine by the bacterial culture. Similar results were obtained with the proline and glycine mutants.

Two other mutants have also been isolated by the method described above—the first is a mutant of *E. coli* W which, unlike the phenylalanine auxotroph from which it was derived, requires very high supplements of phenylalanine for growth. This mutant, however, accumulated ^{14}C-labeled phenylalanine as effectively as the wild-type, and the reason for the very high requirement for phenylalanine is not known. A second mutant (derived from an auxotroph of *E. coli* B requiring histidine, methionine and leucine) is defective in potassium transport, as shown by studies of growth in media containing low potassium (10^{-4} *M*) and measurement of accumulation of ^{42}K (see ref. 12).

The specificity of the defect in these mutants has not yet been fully determined. Complete characterization awaits further isolation of transport-negative mutants.

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[1] E. F. Gale, *J. Gen. Microbiol.*, 1 (1947) 53.
[2] G. N. Cohen and H. V. Rickenberg, *Compt. rend.*, 240 (1955) 2086.
[3] G. N. Cohen and H. V. Rickenberg, *Ann. inst. Pasteur*, 91 (1956) 693.
[4] G. N. Cohen and J. Monod, *Bacteriol. Rev.*, 21 (1957) 169.
[5] J. H. Schwartz, W. K. Maas and E. J. Simon, *Biochim. Biophys. Acta*, 32 (1959) 582.
[6] I. Zabin, A. Kepes and J. Monod, *Biochem. Biophys. Research Commun.*, 1 (1959) 289.
[7] B. D. Davis and E. S. Mingioli, *J. Bacteriol.*, 60 (1950) 17.
[8] L. Gorini and H. Kaufman, *Science*, 131 (1960) 604.
[9] J. Lederberg and E. M. Lederberg, *J. Bacteriol.*, 63 (1952) 399.
[10] B. D. Davis and W. K. Maas, *J. Am. Chem. Soc.*, 71 (1949) 1865.
[11] R. Repaske, *Biochim. Biophys. Acta*, 22 (1956) 189.
[12] M. Lubin and D. Kessel, *Biochem. Biophys. Research Commun.*, 2 (1960) 249.

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Intermittent ultrasonic disruption and localisation of enzymes in acetic acid bacteria

Ultramicroscopic particles, containing a great variety of enzymes, mostly oxidases, can be isolated from disrupted acetic acid bacteria[1–5]. Previously we brought evidence in favour of the view that these enzyme-bearing particles do not exist as such in the

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Abbreviations: TPN, triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

cytoplasm of the acetic acid bacteria, but originate as artefacts from the cytoplasmic membrane[3,4]. This conclusion agrees with the experience of other authors on the origin of similar particles in bacteria as *e.g. Bacillus megaterium*[6], *Staphylococcus aureus*[7] and *Azotobacter vinelandii*[8,9].

In the present communication new evidence is supplied by means of a different technique, which supports the above conclusion. We used the method of MARR AND COTA-ROBLES[8], which is called here "intermittent ultrasonic disruption": at several definite time intervals, the ultrasonic treatment is stopped and samples are withdrawn for the analysis of the properties of the systems under investigations and the study of the kinetics of enzyme release; also, cell-fractions, obtained after a desired sonication time, may be purified and separately submitted to ultrasonic treatment. During sonication the viable cell count decreases faster than the turbidity of the suspension, because the turbidity is occasioned both with the intact cells and the broken-up cell-envelopes or "hulls". An enzyme linked to the hulls will be released from the latter during the ultrasonic disruption procedure at the same rate as the decrease in turbidity. On the other hand, a soluble enzyme, which is originally present in the cytoplasm, will be released into the supernatant at the same rate as the decrease of the viability curve.

For most of our experiments we used the same strain as previously, *Gluconobacter liquefaciens*. It was grown for 2 or 3 days at 30° on a solid medium containing 10 % glucose, 1% yeast extract (Difco), 3% $CaCO_3$ and 2.5% agar. After harvesting, the bacteria were washed twice with 0.01 *M* phosphate buffer, pH 6.2, and finally suspended in a 0.02 *M* solution of the same buffer. The turbidity was adjusted to obtain a reading of approx. 200 in the Klett colorimeter with filter 66. The suspension was disrupted in the 10 kc, 250 W Raytheon Sonic Oscillator at 12° and in air. At the beginning of the sonication and for every 2 or 3 min up to 15 min, a sample was removed (called "total sonicate"), the turbidity measured in the Klett colorimeter with filter 66 and the activity of various enzymes determined as described below. These preparations were separated by centrifugation for 60 min at 6000 × *g* into a "sediment" and a supernatant, called "crude extract". The sediment was used for the estimation of the oxidase activity in the Warburg respirometer at 30°, the vessel containing 1.5 ml of enzyme preparation (the sediment suspended in the original volume of fresh 0.02 *M* phosphate buffer, pH 6.2), 20 μmoles substrate and 40 μmoles phosphate buffer, pH 6.2, in a final volume of 2 ml. Glucose-6-phosphate dehydrogenase activity was determined in the Beckman Spectrophotometer, model DU, in 3 ml containing 0.3 μmole TPN, 10 μmoles Na glucose 6-phosphate, 5 μmoles $MgSO_4$, 110 μmoles Tris–HCl buffer pH 8.5 and 1 ml of either total sonicate or crude extract. The viable cell count was determined by the method of MILES AND MISRA[10].

Several of the enzymes were inactivated to an appreciable extent by the ultrasonic disruption treatment (Fig. 1). These inactivations varied from nearly negligible for galactose oxidase to nearly complete for ethanol oxidase (after 12 min) and D-lactate oxidase (after 15 min).

The galactose oxidase behaved as expected for a "hull"-linked enzyme: its activity was retained on the sediment during sonication in the same way as the decrease in turbidity. For the other enzymes in Fig. 1 this clear-cut picture was complicated by the effect of inactivation. However, we could show that the inactivation occurred mainly on the small particles during sonication. Therefore, a sample was withdrawn from the Raytheon after 4 min of sonic treatment. The crude extract was prepared, which was again sonicated for another 10 min. The 2-ketoglucono-oxidase of the "total sonicate" was inactivated to the extent of 2.6%/min, while the

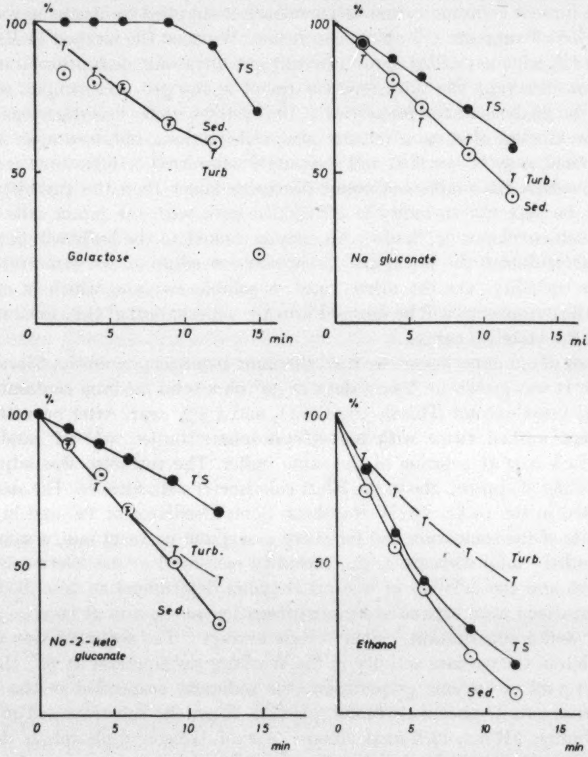

Fig. 1. Behaviour and localisation of various enzymes during the intermittent ultrasonic disruption of *Gluconobacter liquefaciens*. The time axis represents the duration of the ultrasonic treatment in minutes. The results at zero time represent the behaviour of the intact cells and are arbitrarily expressed as 100%. The activity of the oxidases for galactose, sodium gluconate, sodium 2-keto-gluconate and ethanol were determined with the Warburg respirometer (see text). Curve *T*—*T* Turb., change of turbidity of the total sonicate; ●—● TS, activity of the enzyme in the total sonicate (inactivation curve); ⊙—⊙ Sed., activity of the enzyme in the sediment.

same enzyme in the "crude extract" was inactivated to the extent of 2.8%/min. It may thus be concluded that also in the latter cases, the activity is due to the enzymes linked to the "hulls". We found this to be the case for the oxidases of gluconate and 2-ketogluconate, for the enzyme systems for ethanol and D-lactate oxidation in *Gluconobacter liquefaciens* and for the glucose oxidase in *Acetobacter rancens*. When the activity of some of the enzyme systems was tested in the crude extract, it was found to be very weak. All these enzymes were previously found to be linked to the ultramicroscopic particles and to the "ghost" fraction[3,4].

The viable count decreased much faster that the turbidity. After 15 min the latter had decreased by about 50 %, while more than 90 % of the original amount of cells were disrupted. The glucose 6-phosphate dehydrogenase activity was selected as a soluble enzyme because it was not noticeably inactivated by ultrasonic treatment up to 15 min. Nearly all the activity of the total sonicate was present in the crude extract. The enzyme was released from the sediment at the same rate as the killing of the bacteria, as expected for a soluble enzyme (Fig. 2). The soluble TPN-linked ethanol dehydrogenase was unsuitable for this purpose because of the pronounced inactivation; no activity remained after 12 min of sonic treatment.

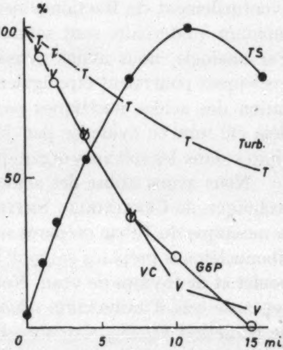

Fig. 2. The behaviour of a soluble enzyme (glucose 6-phosphate dehydrogenase) during the intermittent ultrasonic disruption of *Gluconobacter liquefaciens*. The time axis represents the duration of the ultrasonic treatment in min. The values of turbidity (curve *T*—*T*) and viable count (curve *V*—*V*) at zero time are the results obtained with intact cells and arbitrarily expressed as 100 %. The activity of the glucose 6-phosphate dehydrogenase in the crude extract (○—○ G6P) was maximal after 15 min and this value was taken as 100 %. The curve was inversed in the graph to show the congruence with the viable count. The activity o the dehydrogenase in the total sonicate (●—● TS) was maximal after 11 min and this value was taken as 100 %. Methods as described in the text.

These results bring additional evidence that the enzyme-bearing particles in the acetic acid bacteria are artefacts derived from the breakage of the outer cell envelope, most probably the cytoplasmic membrane. In these experiments, as in those described by Marr and Cota-Robles[8], it appears likely that the cytoplasmic membrane remains tightly joined to the cell wall to form the "hulls" during breakage.

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[1] J. De Ley and A. J. Stouthamer, *Biochim. Biophys. Acta*, 34 (1959) 171.
[2] J. De Ley and J. Schell, *Biochim. Biophys. Acta*, 35 (1959) 154.
[3] J. De Ley and R. Dochy, *Arch. intern. physiol. et biochim.*, 68 (1960) 215.
[4] J. De Ley and R. Dochy, *Biochim. Biophys. Acta*, 40 (1960) 277.
[5] J. De Ley, unpublished results.
[6] C. Weibull, H. Beckman and L. Bergstrom, *J. Gen. Microbiol.*, 20 (1959) 519.
[7] P. Mitchell, *Biochem. Soc. Symposia (Cambridge, Engl.)*, 16 (1959) 73.
[8] A. G. Marr and E. H. Cota-Robles, *J. Bacteriol.*, 74 (1957) 79.
[9] E. H. Cota-Robles, A. G. Marr and E. H. Nilson, *J. Bacteriol.*, 75 (1958) 243.
[10] A. A. Miles and S. S. Misra, *J. Hyg.*, 38 (1938) 732.

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Preliminary Notes

Etude de la précipitation des acides nucléiques par les sels d'ammonium quaternaire

Nous avons entrepris ce travail dans le but d'étudier une solubilisation possible des acides nucléiques dans les solvants organiques en vue d'interventions chimiques et éventuellement de fractionnement. VIOVY[1] a montré que les polyphosphates d'ammonium quaternaire sont solubles dans un certain nombre de solvants organiques. Par analogie, nous avons pensé que les sels d'ammonium quaternaire des acides nucléiques pourraient être également solubles dans ces mêmes solvants. La précipitation des acides nucléiques par le bromure de triméthyl hexadécyl ammonium a déjà été mise en évidence par JONES[2], mais sans étude de la solubilité de ces précipités dans les solvants organiques.

Nous avons utilisé des acides ribonucléiques préparés à partir de levures par la technique de CRESTFIELD, SMITH ET ALLEN[3], des acides ribonucléiques de virus de la mosaïque du Tabac préparés suivant la technique de SCHRAMM[4], des acides désoxyribonucléiques préparés suivant la technique de POUYET[5] à partir d'érythrocytes de poulet et de thymus de veau. Nous avons systématiquement étudié l'action de divers types de sels d'ammonium quaternaire, en particulier toute la série des bromures de triméthyl C_8, C_{10}, C_{12}, C_{14}, C_{16} et C_{18} ammonium. Nous avons observé que les deux premiers de la série ne précipitent pas les acides nucléiques, la précipitation commence avec le bromure de triméthyl C_{12}: d'abord partielle elle devient totale en fonction du temps. La précipitation est totale et immédiate avec les bromures de triméthyl C_{14} et C_{16}. Le bromure de triméthyl C_{18} n'est pas soluble dans l'eau. Les sels d'ammonium quaternaire de courte chaîne (chlorure de triéthyl benzyl ammonium, bromure de tétra méthyl ammonium et hydroxyde de benzyl triméthyl ammonium) ne précipitent pas les acides nucléiques. Le Tableau I donne un résumé des résultats obtenus.

Nous avons particuliérement travaillé avec le bromure de triméthyl hexadécyl ammonium. Si à du RNA en solution aqueuse ou chlorurée, ou à du DNA en solution chlorurée, on ajoute du triméthyl C_{16} ammonium en solution aqueuse, on observe une précipitation immédiate. L'étude de la précipitation en fonction des quantités de sel d'ammonium mises en jeu nous a montré que la précipitation totale s'obtient pour des quantités de sel allant de 1 reste ammonium jusqu'à 4 restes par nucléotide. Avec des quantités plus grandes on observe une inhibition de la précipitation; ce fait a d'ailleurs été récemment signalé par GUERRITORE ET BELLELI[6] mais leurs résultats diffèrent quantitativement des nôtres.

Les composés obtenus ne renferment plus de sodium. Le pourcentage de phosphore diminue: la valeur moyenne obtenue est de 4.4 pour 100 pour le RNA et de 4.3 pour 100 pour le DNA. Les sels d'ammonium quaternaire des acides nucléiques sont solubles

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Abbréviations: RNA, acide ribonucléique; DNA, acide deoxyribonucléique.

dans un certain nombre de solvants organiques. Nous avons particulièrement étudié les solutions dans la diméthylformamide et dans l'éthanol absolu. A partir de ces solutions on peut reprécipiter totalement les acides nucléiques au moyen de solutions concentrées de NaCl. Le produit obtenu, soluble dans l'eau et dans les solutions diluées de NaCl présente le spectre caractéristique des acides nucléiques, les valeurs d'$\varepsilon_{(P)}$ sont sensiblement égales pour les RNA et DNA natifs et précipités à partir des solutions organiques.

TABLEAU I

Sel d'ammonium quaternaire	*RNA*	*DNA*
Triéthyl benzyl Am.	o	o
Triméthyl benzyl Am.	o	o
Tétraméthyl Am.	o	o
Triméthyl C_8 Am.	o	o
Triméthyl C_{10} Am.	o	o
Triméthyl C_{12} Am.	+ lent	+ lent
Triméthyl C_{14} Am.	+	+
Triméthyl C_{16} Am.	+	+
Benzyl diméthyl C_{12} Am.	+	+
Benzyl diméthyl C_{14} Am.	+	+
Benzyl diméthyl C_{16} Am.	+	+
C_{12} Pyridinium	+	+
C_{14} Pyridinium	+	+
C_{16} Pyridinium	+	+
"Désogène"	+	+
"Hyamine"	+	+

+ précipitation totale. o pas de précipitation.

En résumé, nous avons montré, dans ce travail préliminaire, que les acides deloxy et ribonucléiques donnent avec les sels d'ammonium quaternaire de constitution convenable des précipités solubles dans certains solvants non aqueux d'où on peut reprécipiter l'acide nucléique sous forme de sel de sodium. L'étude physique des nucléates d'ammonium quaternaire dans les solvants organiques ainsi que l'étude du pouvoir infectieux du RNA du virus de la mosaïque du Tabac feront l'objet de prochaines notes. Nous pensons que les résultats que nous apportons ouvrent, grâce à la solubilisation dans les solvants organiques, des perspectives intéressantes vers la possibilité d'un fractionnement des acides nucléiques aussi bien que vers leur transformation par certains agents chimiques.

Nous remercions vivement le Dr. Hirth qui nous a donné des RNA de virus de la mosaïque du Tabac et le Dr. Pouyet qui a préparé pour nous les divers DNA utilisés.

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[1] R. Viovy, *Symposium sur les Macromolécules, Wiesbaden, 1959*, Section IV.
[2] A. S. Jones, *Biochim. Biophys. Acta*, 10 (1953) 607.
[3] A. M. Crestfield, K. C. Smith et F. W. Allen, *J. Biol. Chem.*, 216 (1955) 185.
[4] H. Schuster, G. Schramm et W. Zillig, *Z. Naturforsch.*, 11B (1956) 339.
[5] J. Pouyet, communication personnelle.
[6] D. Guerritore et L. Belleli, *Nature*, 184 (1959) 1638.

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Studies on the fractionation of ribonucleic acid

As first shown in this laboratory, DNA can be separated into a series of fractions of regularly graded composition through the fractional dissociation of native or artificially prepared nucleic acid–histone complexes by salt solutions of increasing strength in the presence of a $CHCl_3$ phase[1,2]. The present note explores the application of this method to RNA.

RNA specimens (free of DNA) were prepared from rat liver by a modification of a previously described procedure[3]. The tissue was ground for 1 min in 5 vol. 10^{-4} *M* EDTA, pH 8, and the pulp treated with an equal vol. of 90 % phenol for 1 h at room temp. The aqueous phase, separated by centrifugation (20,000 × *g*, 30 min) and combined with the EDTA washings of the phenol layer, was adjusted to a 2 % potassium acetate concentration and mixed with 2 vol. ethanol. The RNA thus precipitated was dissolved in EDTA and separated into high-molecular RNA insoluble in 10 % NaCl (about 80 % of the total) and into RNA soluble in strong salt solution (15–20 %).

Conversion to the respective histone nucleates was followed by their stepwise extraction, in the presence of $CHCl_3$, with increasing concentrations of NaBr under the conditions otherwise specified previously[2]. The insoluble RNA thus was divided into 8 fractions between 0.5 and 5.2 *M* NaBr, the soluble RNA into 5 fractions between 0.2 and 1.0 *M* NaBr.

The general procedure for the estimation of the nucleotide composition of the fractions has been described[4]; but it appeared of interest also to study the distribution of minor RNA constituents, especially of pseudouridylic acid. For the quantitative determination of the latter, alkaline hydrolysates were subjected to descending 2-dimensional paper chromatography: I, isopropanol–NH_3 [5](72 h); II, isobutyric acid–ammonium isobutyrate[6] (20 h). In I, pseudo uridylic precedes guanylic acid, but is slower than the band containing the other nucleotides; in II, uridylic and guanylic acids run adjacent to each other, whereas pseudouridylic acid is slightly slower. All 5 constituents can thus be separated. The identity of pseudouridylic acid was established by chromatographic comparison with an authentic sample kindly supplied by Dr. W. E. COHN[7] and also through the characteristic spectra at pH 1 and 13[8]. The nature of several minor components also seen on the chromatograms is being investigated.

As concerns the nucleotide composition of the RNA specimens before fractionation, Table I suggests the existence of substantial differences between the RNA insoluble in 10 % NaCl and the soluble RNA, the former exhibiting higher proportions of guanylic and cytidylic acids. The most striking divergence is seen in the proportion of pseudouridylic acid which is much higher in the soluble RNA. This finding is similar to observations on pseudouridine in the microsomal and the non-sedimentable RNA of rat liver[9]. By way of comparison, we have found the NaCl-insoluble RNA isolated from a thymine-requiring strain of *Escherichia coli* to be free of trace components.

Of particular interest are the differences between DNA and RNA as regards the fractional dissociation, under denaturing conditions, of their respective histone

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EDTA, ethylenediaminetetraacetate.

TABLE I

INSOLUBLE AND SOLUBLE RAT LIVER RNA; COMPOSITION AND FRACTIONATION*

Starting preparation	Fraction			Moles/100 moles nucleotide in RNA					Molar ratios		
	No.	NaBr (M)	% of RNA in starting material	A	G	C	U	Ps	$\frac{A+U+Ps}{G+C}$	$\frac{6\text{-}Am}{6\text{-}K}$	$\frac{Ps}{U} \times 100$
Insoluble RNA	Total	—	100	19.6	32.2	29.7	17.3	1.2	0.62	0.97	6.9
	1	0.5	4.0	21.7	29.7	26.8	19.5	2.2	0.77	0.94	11.3
	2–8	0.65–5.2	82.3	18.6–19.5	32.1–33.7	29.0–31.1	16.2–17.9	1.0–1.3	0.59	0.96	7.0
Soluble RNA	Total	—	100	20.5	30.4	28.3	17.4	3.3	0.70	0.95	19.0
	1	0.2	33.0	21.2	29.8	29.0	16.3	3.8	0.70	1.01	23.3
	2–4	0.35–0.75	62.5	21.0–21.4	30.4–31.4	27.2–28.9	15.2–18.3	3.1–3.4	0.69	0.96	19.0
	5	1.0	3.9	21.3	30.8	28.2	17.9	1.8	0.69	0.98	10.0

* A, G, C, U, Ps stand for adenylic, guanylic, cytidylic, uridylic and pseudouridylic acids, respectively. 6-Am denotes the 6-amino nucleotides (A+C), 6-K the 6-keto nucleotides (G + U + Ps). For the sake of brevity, only the range of analytical results is given for Fractions 2–8 of the insoluble RNA and for Fractions 2–4 of the soluble RNA; in these cases the molar ratios represent averages. For the quantitative estimation of Ps, a value of $\varepsilon = 8{,}400$ at 262.5 mμ in 0.1 N HCl was used[8].

nucleates. Not only is the RNA, especially the portion insoluble in salt, more strongly held by histone, requiring, for dissociation, the more effective bromide rather than chloride, but the various fractions, although released characteristically over a wide molarity range, appear to differ less in composition than is observed with DNA. It will be seen in Table I that—in contrast to the DNA fractions exhibiting a gradual transition from the GC to the AT type[1,2]—only Fraction 1 of the insoluble RNA was significantly different in composition from that of the total in having a higher content of adenylic, uridylic and pseudouridylic acids. The 5 fractions yielded by the soluble RNA differed mainly with regard to the concentration of pseudouridylic acid, especially when Fractions 1 and 5 are compared. In all cases, the ratio 6-Am/6-K[10] was close to unity. It should be added that there is some evidence that the fractions, despite similarities in their composition, are distinguished by certain features of the nucleotide arrangement. This, as well as related investigations, will be discussed later in another context.

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[1] E. CHARGAFF, C. F. CRAMPTON AND R. LIPSHITZ, *Nature*, 172 (1953) 289.
[2] C. F. CRAMPTON, R. LIPSHITZ AND E. CHARGAFF, *J. Biol. Chem.*, 211 (1954) 125.
[3] R. LASKOV, E. MARGOLIASH, U. Z. LITTAUER AND H. EISENBERG, *Biochim. Biophys. Acta*, 33 (1959) 247.
[4] D. ELSON, T. GUSTAFSON AND E. CHARGAFF, *J. Biol. Chem.*, 209 (1954) 285.
[5] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
[6] B. MAGASANIK, E. VISCHER, R. DONIGER, D. ELSON AND E. CHARGAFF, *J. Biol. Chem.*, 186 (1950) 37.
[7] W. E. COHN, *J. Biol. Chem.*, 235 (1960) 1488.
[8] F. F. DAVIS AND F. W. ALLEN, *J. Biol. Chem.*, 227 (1957) 907.
[9] D. B. DUNN, *Biochim. Biophys. Acta*, 34 (1959) 286.
[10] D. ELSON AND E. CHARGAFF, *Biochim. Biophys. Acta*, 17 (1955) 367.

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Effect of magnesium on the amino acid-dependent pyrophosphate exchange catalysed by "RNA-low" systems

Recent evidence suggests that the stability of the leucine-activating enzyme present in rat-liver pH-5.0 fractions[2], is due to a special association between enzyme and "soluble" RNA, conferring protection upon labile thiol groups concerned both in pyrophosphate exchange, and in the transfer of [^{14}C]leucine to "soluble" RNA. The instability of activating enzymes found in "RNA-low fractions" obtained from protamine-treated rat-liver extracts[1,3], probably results from the interaction or

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Abbreviations: RNA, ribonucleic acid; ATP, adenosine triphosphate; AMP, adenosine 5′-phosphate; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

removal of "soluble" RNA with protamine. Activating enzymes, precipitated with yeast RNA[3], are as unstable as those occurring in "RNA-low fractions".

It has now been observed that unstable activating enzymes are considerably stimulated by Mg^{++} in concentrations above 5 m*M*. At these levels the stable leucine-activating enzyme in the pH-5.0 fraction shows little stimulation (Table I), and often depression. This amino acid-dependent pyrophosphate exchange observed at high Mg^{++} levels is very readily and reproducibly depressed by AMP, in a manner suggesting that the AMP is involved in the reaction mechanism. At lower Mg^{++} concentrations the depressing effect of AMP is encountered less reproducibly, particularly with the leucine-activating enzyme of the pH-5.0 fraction. It is intended to re-examine this phenomenon using [^{14}C]AMP[4], to ensure that these inhibitory effects of AMP are not misleading.

TABLE I

THE EFFECT OF Mg^{++} AND AMP UPON AMINO ACID-DEPENDENT PYROPHOSPHATE EXCHANGE CATALYSED BY RAT-LIVER PREPARATIONS

Mg^{++}	*Pyrophosphate exchange (mμmoles/min/mg protein)*							
	"RNA-low" fraction				*pH-5.0 fraction*			
	Leucine		*Lysine*		*Leucine*		*Lysine*	
	−AMP	*+AMP*	*−AMP*	*+AMP*	*−AMP*	*+AMP*	*−AMP*	*+AMP*
1 m*M*	23	5	0	0	16	14	0	0
5 m*M*	77	66	15	7	52	56	14	4
15 m*M*	147	79	131	64	60	42	70	32
30 m*M*	82	43	246	116	46	16	104	58

The test system contained in a final volume of 0.55 ml: 3 μmoles sodium ATP, 1.5 μmoles sodium [^{32}P]pyrophosphate, 0.5 μmole amino acid, 50 μmoles Tris buffer (pH 7.2), 0.5 mg protein, and 8.7 μmoles sodium AMP (Sigma) or a compensating amount of NaCl in the tubes without AMP. Mg^{++} was added as indicated. Incubation was for 5 min at 37°. Separation of ATP from pyrophosphate in the presence of AMP by our method[3] required about 400 mg equivalent of dry charcoal. Each assay was accompanied by an amino acid-free blank, which remained constant throughout the series, and which has been subtracted from the data presented here.

When lysine is used as substrate the results are clear-cut and reproducible, provided that freshly-made preparations are employed, since the enzyme concerned is very labile. When leucine is used as substrate, the results are more variable, and seem to alter with slight changes in those isolation procedures which might be expected to influence the association between "soluble" RNA and enzyme.

The transfer of [^{14}C]lysine to "soluble" RNA catalysed by the pH-5.0 fraction is only one half to one third that of the transfer obtained with [^{14}C]leucine. When, however, this transfer is examined in "recombined systems" it is found that the transfer of [^{14}C]lysine is equal to that of [^{14}C]leucine. This suggests that a faulty association exists between the enzyme in the pH-5.0 fraction and the "soluble" RNA, parallel with its instability, and type of response to Mg^{++} and AMP. Activating enzymes for valine and threonine, showing similar properties are found in these pH-5.0 fractions[3].

Recently, ALLEN *et al.*[5] have suggested that thiol groups may participate in the mechanism of amino acid activation. The results reported here indicate that an aminoacyl thioester may be involved as an intermediate, the mechanism of its formation being similar to that of acetyl-coenzyme A[4]. If this is so, then free AMP must appear in the system before the transfer of the activated amino acid to the "soluble" RNA. This transfer is known to be reversed by free AMP (and pyrophosphate), suggesting that free AMP appears after the transfer has taken place (for discussion, see LIPMANN *et al.*[6]). The dilemma can be resolved if we assume that the amino-acyl thioester and amino-acyl ribose ester bonds are in equilibrium with each other, perhaps through an intermediary complex containing Mg^{++}. In the stable amino acid-activating systems this hypothetical complex may effect the "coupling" of thioester bond synthesis to the transfer of the activated amino acid to "soluble" RNA, the formation of thioester bonds being controlled through the response of the thioester-generating system to Mg^{++}. This controlling or "coupling" action may represent a highly interesting property of "soluble" RNA.

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[1] P. HELE, *Biochem. J.*, 76 (1960) 19 P.
[2] M. B. HOAGLAND, M. L. STEPHENSON, F. J. SCOTT, L. I. HECHT, AND P. C. ZAMECNIK, *J. Biol. Chem.*, 231 (1958) 241.
[3] P. HELE AND L. R. FINCH, *Biochem. J.*, 75 (1960) 352.
[4] P. BERG, *J. Biol. Chem.*, 222 (1956) 991.
[5] E. H. ALLEN, E. GLASSMAN AND R. S. SCHWEET, *J. Biol. Chem.*, 235 (1960) 1061.
[6] F. LIPMANN, W. C. HÜLSMANN, G. HARTMAN, H. G. BOMAN AND G. ACS, *Symposium on Enzyme Reaction Mechanisms*, Philadelphia, 1959, Wistar Institute of Anatomy-Biology, p. 75.

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The enzymic synthesis of thymidine diphosphate glucose and thymidine diphosphate rhamnose

Recently a large variety of thymidine-linked sugar nucleotides have been isolated from microorganisms[1–4]. The sugars include rhamnose[1,4], mannose[4] and a variety of unidentified sugars[2,3]. We wish to report evidence for the enzymic formation of thymidine diphosphate glucose and its conversion to thymidine diphosphate rhamnose in cell-free extracts of *Pseudomonas aeruginosa* (ATCC 7700). This organism has been shown to secrete a rhamnose-containing lipid into the culture medium[5,6].

Cells were grown in a 3% glycerol–mineral salts medium[6] for 100 h at 32°. The cells were harvested by centrifugation and washed with 0.05 *M* Tris–0.001 *M* EDTA, pH 8. The washed cells were suspended in 0.05 *M* Tris–0.01 *M* $MgCl_2$–0.001 *M* EDTA, pH 8, and ruptured by 18-min treatment in a 10 kc magnetostriction oscillator. Intact cells and large particles were removed by centrifugation at 20,000 × *g* for 20 min.

When the sonic extract was incubated with TTP and α-glucose 1-phosphate, followed by isolation of the nucleotides by adsorption on charcoal and chromatography in the neutral ethanol–ammonium acetate solvent[7], a new nucleotide spot

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; TMP, thymidine 5′-phosphate; TDP, thymidine 5′-diphosphate; TTP, thymidine 5′-triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate.

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with R_{TMP} of 1.26 was observed. This compound was not formed when α-mannose 1-phosphate or α-galactose 1-phosphate were used instead of α-glucose 1-phosphate.

When ^{14}C-labelled α-glucose 1-phosphate was used, the isolated compound contained 1 μmole sugar/μmole nucleotide. After mild-acid hydrolysis (pH 2, 100°, 20 min) only part of the sugar could be accounted for as glucose either by chromatography or by enzymic assay with glucose 6-phosphate dehydrogenase and hexokinase. Depending on the time of incubation preparations containing 0.3–0.8 μmole glucose/μmole nucleotide were found. The remainder of the nucleotide was linked to sugars which have not been positively identified.

A somewhat purified enzyme was prepared by removal of nucleic acid with protamine, followed by ammonium sulfate fractionation between 55 and 75 % satn.

A typical reaction mixture contained 75 μmoles Tris, 15 μmoles $MgCl_2$, 1.5 μmoles EDTA, 5 μmoles cysteine, 0.1 μmole adenosine 5'-triphosphate, 10 μmoles phosphoenolpyruvate, 5 μmoles TTP, 4 μmoles α-glucose 1-phosphate, 0.2 mg lactic dehydrogenase containing pyruvic kinase, and 13 mg of the ammonium sulfate fraction in a final volume of 3.3 ml, pH 8.0. The pyruvic kinase and catalytic amounts of adenosine 5'-triphosphate were added to regenerate any TTP which was hydrolyzed by contaminating enzymes. After 1-h incubation at 37° protein was removed by precipitation with $HClO_4$ and the nucleotides isolated by adsorption on charcoal, followed by paper chromatography in the neutral ethanol–ammonium acetate solvent. 1.7 μmoles of thymidine diphosphate glucose were obtained.

The isolated material pooled from several preparations had a typical thymidine spectrum both in neutral and alkaline solution. The ratio of thymidine to acid labile phosphate to total phosphate was 1:0.92:2.1.

After mild acid hydrolysis (pH 2, 100°, 20 min) all the nucleotide was converted to a compound with the mobility of TDP. Mild-acid hydrolysis released 0.8 μmole glucose/μmole of thymidine (determined enzymically with hexokinase and glucose 6-phosphate dehydrogenase).

When treated with snake-venom nucleotide pyrophosphatase 0.8 μmole α-glucose-1-phosphate was formed/μmole of nucleotide (determined with crystalline muscle phosphoglucomutase and glucose 6-phosphate dehydrogenase), indicating that the glucose is linked in the α-configuration. These data show that the new nucleotide has the structure of thymidine diphosphate glucose.

When [^{14}C]glucose-labelled thymidine diphosphate glucose was incubated with the sonic extract which had been centrifuged at 100,000 × *g* for 1 h to remove particulate material, in the presence of a DPNH-generating system (DPN, ethanol and crystalline yeast alcohol dehydrogenase) a sugar could be isolated from the nucleotide fraction which had the mobility of authentic rhamnose when chromatographed using either butanol–pyridine–water (6:4:3) or butanol–acetic acid–water (52:13:35) as the solvent. After addition of rhamnose carrier, rhamnose phenylosazone was prepared and crystallized to constant specific activity. No rhamnose was formed in the absence of a DPNH-generating system. When uridine diphosphate glucose was used as the substrate, no rhamnose was formed.

The enzymic synthesis of thymidine diphosphate rhamnose is formally similar to the enzymic synthesis of guanosine diphosphate fucose from guanosine diphosphate mannose, described by GINSBURG[8]. Attempts are in progress to determine the intermediates in the enzymic synthesis of thymidine diphosphate rhamnose.

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[1] R. OKAZAKI, *Biochem. and Biophys. Research Communs.*, 1 (1959) 34.
[2] J. L. STROMINGER AND S. S. SCOTT, *Biochim. Biophys. Acta*, 35 (1959) 552.
[3] R. OKAZAKI, T. OKAZAKI AND Y. KURIKI, *Biochim. Biophys. Acta*, 38 (1960) 384.
[4] J. BADDILEY AND N. L. BLUMSON, *Biochim. Biophys. Acta*, 39 (1960) 376.
[5] F. G. JARVIS AND M. J. JOHNSON, *J. Am. Chem. Soc.*, 71 (1949) 4124.
[6] G. HAUSER AND M. L. KARNOVSKY, *J. Bacteriol.*, 68 (1957) 645.
[7] A. C. PALADINI AND L. F. LELOIR, *Biochem. J.*, 51 (1952) 426.
[8] V. GINSBURG, *J. Am. Chem. Soc.*, 80 (1958) 4426.

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Hydrogen–deuterium exchange of small peptides in aqueous solution

Measurements of the rate of hydrogen–deuterium exchange in non-intramolecularly hydrogen-bonded peptides and peptide analogs in aqueous solution will greatly facilitate the quantitative interpretation[1,2] of H–D exchange in proteins and polypeptides as measured with the exchange technique of LINDERSTRØM-LANG[3]. A recent communication[4] has reported some preliminary infrared measurements of the H–D exchange in N-methylacetamide. Such studies have now been extended to some simple peptides.

To measure the rate of the exchange reaction of the peptide group

$$\text{–CO–NH–} + D_2O \rightarrow \text{–CO–ND–} + HDO$$

125 μl of a 2 % solution of the peptide in H_2O was lyophilized in a small test tube over conc. H_2SO_4. Subsequently a 1-ml syringe with a spring-loaded piston was filled with approximately 230 μl 99.8 % D_2O containing either small amounts of buffer acids and salts or of HCl. The lyophilized peptide was quickly dissolved by emptying the syringe into the test tube via a special two-way valve with a 0.3-mm bore and pumping the solution up and down a few times. Immediately following this operation the valve was turned, allowing the reaction mixture to be forced directly into a 0.10-mm optical cell with fluorite windows placed in the beam of a Perkin-Elmer Model 13U Infrared Spectrometer arranged for single-beam operation with a fluorite prism. In order to keep the cell temperature constant, thermospacers at 22° were used. The exchange reaction was followed by recording the increasing transmission at 1580 cm^{-1} (essentially the disappearance of the amide II band of the protonated peptide group). In the majority of cases it was possible to start following the course of the exchange reaction approximately 10 sec after its inception. "pD" was determined in the combined reaction mixtures from duplicate runs as described elsewhere[5].

With dipeptides (Gly–Gly, Gly–Ala, Gly–Leu, Ala–Gly, Ala–Ala) the exchange reaction of the peptide group was found to follow first-order kinetics up to at least 75 % conversion. (Amine and carboxyl hydrogen atoms exchange at a rate too fast to be measured by this technique.) With tripeptides (Gly–Gly–Gly, Ala–Gly–Gly, Leu–Gly–Gly) the course of the exchange reaction as recorded did not in general correspond to simple first-order kinetics, but the exchange data could be accounted for within experimental error when it was assumed that the exchange reactions of the two peptide groups in the tripeptide molecule proceeded according to different first-order rate laws. At some pD's it was possible to assign definite values to the two rate constants involved. Table I summarizes some of the results obtained.

The variation of the rate constants in Table I with pD resembles that obtained previously with N-methylacetamide[4] in that there is a minimum in rate and that on the acid side of the rate minimum first-order acid catalysis is observed. However, instead of the minimal rate occurring around pD 5.4 in the case of N-methylacetamide it occurs in Gly–Gly at pD about 2.4 and in Ala–Gly–Gly at pD about 2.2 (N-terminal peptide group) and pD about 3.0 (C-terminal peptide group). A detailed discussion of the assignment of the measured rate constants to the two peptide groups in Ala–Gly–Gly in Table I will appear in *Compt. rend. trav. Lab. Carlsberg*. The exchange behaviour of the di- and tripeptides investigated relative to that of N-methylacetamide is in apparent agreement with the exchange mechanism proposed by BERGER and coworkers[6], when the effects of electron-withdrawing substituents on the amide group is taken into account.

TABLE I

FIRST-ORDER RATE CONSTANTS OF THE H–D EXCHANGE REACTIONS IN GLY–GLY AND ALA–GLY–GLY

Gly–Gly		*Ala–Gly–Glv*		
			N-terminal -CO–NH-	*C-terminal -CO–NH-*
pD	*min⁻¹*	*pD*	*min⁻¹*	*min⁻¹*
1.10	4.1	1.14	1.4	
1.33	2.0	1.22	0.97	
1.95	0.58	1.34	0.86	
2.01	0.60	1.67	0.45	5.6
3.14	1.06	2.03	0.30	2.6
3.84	3.6	3.38		0.71
		4.14		0.91

Our results do not appear to be compatible with some measurements by the Linderstrøm-Lang exchange technique of the rate of H–D exchange in deuterated triglycine at 0° and pH 3 in water. It was reported[3] that 80 % of the peptide hydrogen atoms had exchanged within the first 0.8 min following dissolution of the peptide in water. In contrast, the data in Table I and additional data on triglycine suggest that this H–D exchange in triglycine should be considerably slower. This discrepancy between the two methods will be briefly discussed in the following note.

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[1] W. P. Bryan and S. O. Nielsen, *Compt. rend. trav. Lab. Carlsberg*, in preparation.
[2] W. P. Bryan and S. O. Nielsen, *Biochim. Biophys. Acta*, 42 (1960) 552.
[3] K. Linderstrøm-Lang, in A. Neuberger, *Symposium on Protein Structure*, Methuen, London 1958, p. 23.
[4] S. O. Nielsen, *Biochim. Biophys. Acta*, 37 (1960) 146.
[5] K. Mikkelsen and S. O. Nielsen, *J. Phys. Chem.*, 64 (1960) 632.
[6] A. Berger, A. Loewenstein and S. Meiboom, *J. Am. Chem. Soc.*, 81 (1959) 62.

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Hydrogen–deuterium exchange of poly-DL-alanine in aqueous solution

It has been concluded from infrared spectra of films of PDLA that it exists in aqueous solution in the α form[1]. Assuming an α-helical model, Berger and Linderstrøm-Lang were able to account for most of their H–D exchange results obtained with PDLA in aqueous solution[2,3]. As the H–D exchange mechanism of a polypeptide helix would be of considerable interest, the present study was undertaken in an attempt to clarify the exchange mechanism of PDLA in aqueous solution.

The experimental method was essentially that described in the preceding note[4]. The same PDLA preparation used by Berger and Linderstrøm-Lang[2] (containing approximately 30 residues per molecule, DP = 30) was dissolved in dilute NaOH and lyophilized over conc. H_2SO_4 to remove a pyridine contaminant. 99.8 % D_2O containing varying small amounts of HCl, sodium citrate and citric acid was added to give a solution containing 1 % PDLA, and the exchange reaction (A) was followed at 1550 cm^{-1}. Using an analogous procedure, the course of the exchange reaction (B) between deuterated PDLA and H_2O was followed by recording the decreasing infrared transmission of the reaction mixture at 2570 cm^{-1} against time[5]. Thermospacers maintained the cell temperature at 10° or 22°.

The recorded infrared transmission T_t approached asymptotically a constant value $T\infty$. When plotting loglog $(T\infty/T_t)$ (1550 cm^{-1}) or loglog $(T_t/T\infty)$ (2570 cm^{-1}) against time our results in typical cases gave linear plots up to at least 75 % of total conversion. From the slopes of these plots apparent first-order rate constants were calculated, and some of these are presented in Table I.

Our results do not appear to be compatible with those of Berger and Linderstrøm-Lang[2] obtained with PDLA (DP = 30) at 0°, 10° and 20°. None of their exchange curves, giving degree of H–D exchange as a function of time, could be fitted to one exponential decay function as is the case with the data summarized in Table I. It has already been reported[4] that the exchange technique of Linderstrøm-Lang[6] does not give good agreement with the present infrared technique in measurements of the rate of H–D exchange in triglycine. This situation suggested to us that the exchange technique of Linderstrøm-Lang might indicate too high initial rates of H–D exchange. Preliminary control experiments have supported this suggestion in demonstrating that in some instances H–D exchange occurs during the sublimation involved. This phenomenon may account for the deviation of the exchange curves of Berger and Linderstrøm-Lang[2] from simple first-order curves. In this connection it should be pointed out that at a given pH and temperature their experimental points, neglecting zero-time values, fall within experimental

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Abbreviation: PDLA, poly-DL-alanine.

error on a simple first-order exchange curve. Agreement exists between the rate constants calculated from these curves and the rate constants presented in Table I. A detailed discussion of this and the following points will appear in *Compt. rend. trav. Lab. Carlsberg*.

TABLE I

FIRST-ORDER RATE CONSTANTS OF THE H–D EXCHANGE OF PDLA

PDLA (DP = 30)			
22°		*10°*	
pD	k_A *(min⁻¹)*	*pD*	k_A *(min⁻¹)*
1.64	1.27	2.07	0.23
2.37	0.37	4.67	0.21
2.56	0.26		
3.69	0.144		
4.62	0.58		
PDLA (DP = 20), 22°			
pD	k_A *(min⁻¹)*	*pH*	k_B *(min⁻¹)*
2.24	0.48	1.93	0.50
4[illegible]	0.68	4.12	0.65

In view of the present results as well as recent infrared spectral work[7] it would appear that the question whether PDLA exists in aqueous solution as an α-helix would have to be reconsidered. The following observations made during the course of the present investigation weigh against a substantial fraction of PDLA being in the α-helical form in aqueous solution between 0° and 22°. (1) The H–D exchange of PDLA behaves like a simple first-order reaction. (2) The exchange rate is of the same order of magnitude as that found in simple di- and tripeptides[4]. (3) Additions of urea[2] and dioxane have only a small effect on the exchange rate. (4) The rate of H–D exchange in poly-α-L-glutamic acid in solution is very slow under conditions[8] where helix formation occurs.

We are greatly indebted to the late Professor K. LINDERSTRØM-LANG, under whose inspiring leadership this investigation was initiated. A gift of poly-α-L-glutamic acid from Professor R. LUMRY is gratefully acknowledged.

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[1] A. ELLIOTT, *Nature*, 170 (1952) 1066.
[2] A. BERGER AND K. LINDERSTRØM-LANG, *Arch. Biochem. Biophys.*, 69 (1957) 106.
[3] K. LINDERSTRØM-LANG, *Acta Chem. Scand.*, 12 (1958) 851.
[4] S. O. NIELSEN, W. P. BRYAN AND K. MIKKELSEN, *Biochim. Biophys. Acta*, 42 (1960) 550.
[5] S. O. NIELSEN, *Biochim. Biophys. Acta*, 37 (1960) 146.
[6] I. M. KRAUSE AND K. LINDERSTRØM-LANG, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, 29 (1955) 367.
[7] A. ELLIOTT, *Advances in Spectroscopy*, 1 (1959) 214.
[8] P. DOTY, A. WADA, J. T. YANG AND E. R. BLOUT, *J. Polymer Sci.*, 23 (1957) 851.

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Purification du chymotrypsinogene-B du boeuf

Le chymotrypsinogène-B (ChTg-B), dont les proportions dans le suc pancréatique de boeuf sont égales[1] à celles du chymotrypsinogène-A de NORTHROP ET KUNITZ[2], a été cristallisé depuis plusieurs années[3–5]. De nombreux travaux, encouragés par l'apparente homogénéité électrophorétique du produit cristallisé[6], ont été consacrés à l'étude de ses propriétés physicochimiques[5,6], de son activation[7] et de sa structure[8–10]. Les études de structure ayant toutefois montré que le produit n'était pas homogène, même après une purification ultérieure, nous avons cherché à mettre au point une technique satisfaisante pour la préparation d'un précurseur dont l'intérêt est évident.

Notre procédé de purification est basé sur le fait que ChTg-B, protéine acide de point isoélectrique 5.2, peut être séparé des principaux composés qui l'accompagnent (ChTg-A et trypsinogène), grâce à une solubilité moindre dans le sulfate d'ammonium à 0.4 de saturation (SO_4Am_2) et une élution plus aisée au cours d'une chromatographie par échange de cations. Toutes les opérations doivent être effectuées à froid et à un pH légèrement acide, sous peine de modifier la structure du précurseur et d'abaisser son activité potentielle. Les principales étapes de la purification ont été contrôlées en mesurant individuellement, après activation trypsique à 0°, les activités chymotrypsiques A et B, au moyen de l'acétyl-L-tyrosine éthylester et de l'acétyl-L-tryptophane éthylester[1]. Les activité spécifiques ont été déterminées à l'aide de l'acétyl-L-tyrosine éthylester, après activation à 35° (ref. 11).

2 kg de pancréas de boeuf sont extraits par SO_4H_2 0.25 N selon la méthode classique décrite par KUNITZ pour ChTg-A[2]. L'extrait limpide (rapport ChTg-B/ChTg-A, 0.43; teneur en protéines totales, 1.0–1.5 %) est précipité à 0° par addition de SO_4Am_2 saturé jusqu'à une concentration de 0.8 satn. Le précipité est dissous dans HCl 10^{-3} M (teneur en protéines, 2.0 %). Le nouveau précipité obtenu à 0° entre 0.0 et 0.4 satn. est dissous dans HCl 10^{-3} M, dialysé contre cet acide et lyophilisé (rapport ChTg-B/ChTg-A, 1.4; rendement en ChTg-B, 84 %.

Le produit lyophilisé (0.6 g), dissous dans du citrate 0.05 M, pH 4.2 (20 ml), est chromatographié dans une colonne de carboxyméthylcellulose équilibrée avec ce tampon (Fig. 1, diagramme de gauche). Après la sortie d'un petit pic inactif, ChTg-B est élué par du citrate 0.05 M, pH 4.6 en un pic presque symmétrique mais hétérogène. On dialyse et lyophilise la ou les fractions dont l'activité est 2.5–2.6. On chromatographie à nouveau les autres fractions dans les mêmes conditions (Fig. 1, diagramme de droite). Les fractions d'activité 2.5–2.6 sont réunies aux précédentes, après dialyse et lyophilisation. Celles dont l'activité est inférieure, sont définitivement rejetées. Rendement global en ChTg-B, 38 %; taux de purification, 7.

Afin d'évaluer l'homogénéité du produit d'activité 2.5–2.6, un échantillon est soumis à une chromatographie d'équilibre sur DEAE-cellulose à pH 8.0 (Fig. 2). Bien que la dialyse prolongée à laquelle il est indispensable de procéder* avant chromatographie fasse tomber l'activité à 2.2, même en présence de diisopropylfluorophosphate $0.7 \cdot 10^{-4}$ M, le diagramme de la Fig. 2 révèle une homogénéité satisfaisante. En utilisant des phosphates de molarités différentes, le pic de ChTg-B est déplacé dans un sens ou dans l'autre. Mais il reste toujours à peu près homogène.

Abbréviation: ChTg, chymotrypsinogène; SO_4Am_2, sulfate d'ammonium; DEAE-, diethylaminoethyl.

* Quand la solution de ChTg-B est simplent amenée à pH 8.0 avec de la soude, l'augmentation de force ionique est suffisante pour empêcher l'adsorption de la protéine sur DEAE-cellulose.

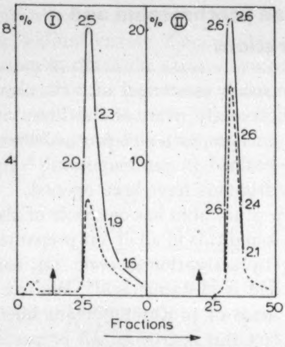

Fig. 1. Chromatographie du chymotrypsinogène-B sur carboxyméthylcellulose. Le tampon d'équilibrage (citrate 0.05 *M* pH 4.2) est remplacé par du citrate 0.05 *M* pH 4.6 au moment indiqué par la flèche. En trait plein: courbe de l'activité potentielle chymotrypsique en %. En pointillé, courbe des protéines en %. Colonne 3.0 × 9.0 cm. Débit, 60 ml. Volume des fractions, 10 ml. Temp., 5°. A gauche, précipité 0.0–0.4 satn. (voir le texte). A droite, nouvelle chromatographie des fractions dont l'activité est inférieure à 2.5 dans le diagramme de gauche.

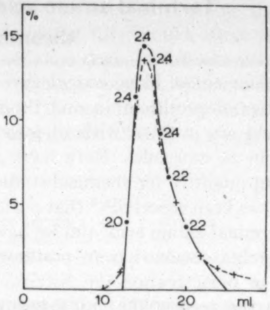

Fig. 2. Chromatographie d'équilibre du chymotrypsinogène-B sur DEAE-cellulose. Colonne (0.9 × 15 cm) de DEAE-cellulose équilibrée avec du phosphate 0.05 *M* pH 8.0, 0.7 10^{-4} *M* en diisopropylfluorophosphate. Solution (1.3 ml) de ChTg-B (20 mg). Activité spécifique initiale, 2.5–2.6; après une dialyse de 24 h à pH 8.0, 2.2. Elution avec le tampon d'équilibrage. Les conventions d'écriture et de dessin sont celles de la Fig. 1.

Le ChTg-B ainsi préparé est totalement inactif. Il possède, comme le ChTg-A[12], 1 résidu N-terminal de 1/2 cystine par mole. Aucun autre résidu N-terminal ne peut y être décelé, ce qui est un signe supplémentaire d'homogénéité. L'étude de sa séquence C-terminale et de sa composition en amino acides est en cours.

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[1] P. J. KELLER, E. COHEN ET H. NEURATH, *J. Biol. Chem.*, 233 (1958) 344.
[2] J. H. NORTHROP, M. KUNITZ ET R. M. HERRIOTT, *Crystalline Enzymes*, 2 ed., Columbia University Press, New York, 1948.
[3] M. LASKOWSKI, *J. Biol. Chem.*, 166 (1946) 555.
[4] C. K. KEITH, A. KAZENKO ET M. LASKOWSKI, *J. Biol. Chem.*, 170 (1947) 227.
[5] E. L. SMITH, D. M. BROWN ET M. LASKOWSKI, *J. Biol. Chem.*, 191 (1951) 639.
[6] V. KUBACKI, K. D. BROWN ET M. LASKOWSKI, *J. Biol. Chem.*, 180 (1949) 73.
[7] K. D. BROWN, R. E. SHUPE ET M. LASKOWSKI, *J. Biol. Chem.*, 173 (1948) 99.
[8] B. KASSEL, *Federation Proc.*, 18 (1959) 257.
[9] B. KASSEL ET M. LASKOWSKI, *Federation Proc.*, 19 (1960) 332.
[10] J. A. GLADNER ET H. NEURATH, *J. Biol. Chem.*, 206 (1954) 911.
[11] C. H. W. HIRS, *J. Biol. Chem.*, 205 (1953) 93.
[12] F. R. BETTELHEIM, *J. Biol. Chem.*, 212 (1955) 235.

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Terminal amino acids of bovine prothrombin and thrombin preparations

The phenomena of blood coagulation are fundamentally concerned with the chemical properties of prothrombin and thrombin. Until recently most of the literature in that field was written without information on the properties of prothrombin and thrombin as molecules. Both have now been obtained in purified form[1,2] and in sufficient quantity for chemical studies, and new frontiers have been opened.

It has been reported[3,4] that purified bovine prothrombin has one mole of alanine as N-terminal amino acid, and we have now also found this in all of our preparations. In thrombin obtained from prothrombin either by activation in conc. aq. sodium citrate or bioactivation the N-terminal amino acid is glutamic acid[5]. We can also confirm the reports that no C-terminal amino acids of prothrombin are liberated with the use of carboxypeptidase A or B[3,4]. In fact this is true for all preparations we have studied.

In further work we studied the C-terminal amino acid composition of prothrombin and thrombin by using the ammonium thyocyanate method of TIBBS[6]. In thrombin the C-terminal amino acid is isoleucine. This conclusion is based on chromatography of the hydantoin with five different solvents, and in addition the amino acid in the separated hydantoin was uncoupled and identified by chromatography in three different solvents. To obtain the free amino acid the hydantoin was placed in 0.25 N $Ba(OH)_2$ and refluxed for 48 h. The barium was precipitated with CO_2 and removed. The amino acid was taken up from the dried supernatant solution in acidulated acetone and chromatographed.

In the case of the bovine prothrombin from which the thrombin preparations were made we find that the C-terminal amino acids may not be the same, but depend upon the isolation procedure. Altogether we used three different methods, each of which give homogeneous prothrombin as observed with an analytical ultracentrifuge. In two of these procedures the prothrombin obtained contains the C-terminal amino acids tyrosine and glycine. Accordingly there must be at least two open chains in the molecule. In another there is only serine, but in all of them the N-terminal amino acid is alanine. Thus, the difference is specifically at the C-terminal end(s).

The preparation[1] obtained by adsorption on $Mg(OH)_2$ contains tyrosine and glycine. If this preparation is chromatographed with IRC-50 (XE-64) as described[7], the C-terminal amino acids are also tyrosine and glycine, but if it is chromatographed on DEAE-cellulose by methods to be described later, there is only one C-terminal amino acid and it is serine. Although the latter prothrombin has approximately the same sedimentation coefficient by ultracentrifuge analysis as the other two it must be altered by the chromatography with cellulose. The preparative procedure can thus make a difference in the chemical structure, and it is interesting that the activation characteristics of prothrombin made in this way are also unique. Whereas the other two preparations become thrombin in conc. aq. sodium citrate or aq. protamine sulfate the prothrombin made with DEAE-cellulose does not, even though it converts to thrombin very well in the two-stage analytical reagents.

Perhaps the prothrombin obtained from plasma which is first passed through

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Abbreviation: DEAE-, diethylaminoethyl-.

a Seitz filter[8] is altered by the filtration and therefore does not become thrombin in aq. sodium citrate. Conceivably the cellulose in the filter could affect the prothrombin in much the same way as the DEAE-cellulose does. In the activation with sodium citrate or protamine sulfate only prothrombin is needed. When it does not activate under those conditions the basis is the difference in the structure of the prothrombin molecule itself, and has nothing to do with the absence of a hypothetical essential procoagulant.

TABLE I

TERMINAL AMINO ACIDS IN BOVINE PROTHROMBIN AND THROMBIN

Preparation	*N-terminal*	*C-terminal*
Thrombin	Glutamic acid	Isoleucine
Prothrombin Mg(OH)$_2$ only	Alanine	Tyrosine Glycine
Prothrombin Mg(OH)$_2$ and IRC-50	Alanine	Tyrosine Glycine
Prothrombin Mg(OH)$_2$ and DEAE-cellulose	Alanine	Serine

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[1] W. H. SEEGERS, *Record Chem. Progr.*, 13 (1952) 143.
[2] W. H. SEEGERS, W. G. LEVINE AND R. S. SHEPARD, *Can. J. Biochem. Physiol.*, 36 (1958) 603.
[3] K. D. MILLER, *State Dept. Health, Ann. Rept. Div. Labs. Research*, (1957) 44.
[4] S. MAGNUSSON, *Acta Chem. Scand.*, 12 (1958) 355.
[5] W. H. SEEGERS, G. CASILLAS, R. S. SHEPARD, W. R. THOMAS AND P. HALICK, *Can. J. Biochem. Physiol.*, 37 (1959) 775.
[6] J. TIBBS, *Nature*, 168 (1951) 910.
[7] K. D. MILLER, *J. Biol. Chem.*, 235 (1958) 987.
[8] R. GOLDSTEIN, A. LE BOLLOCH, B. ALEXANDER AND E. ZONDERMAN, *J. Biol. Chem.*, 234 (1959) 2857.

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Studies on a paraffin-utilizing pseudomonad

A strain of pseudomonad isolated from oily mud on the underside of a motor-cycle gear box was found to grow with any one of a variety of paraffin hydrocarbons as sole carbon source. The best growth was obtained using octadecane as carbon source in a medium containing salts, buffered to pH 7, with $(NH_4)_2SO_4$ as nitrogen source.

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Manometric uptake of O_2 following addition of octadecane was rapid when NH_4^+ was also present. In absence of NH_4^+ the rate of uptake was not significantly different from that in absence of substrate. Cells depleted in absence of ammonia, but with substrate, showed a lag of about 30 min before subsequent O_2 uptake following addition of $(NH_4)_2SO_4$, but cells starved with ammonia present oxidized octadecane without lag when it was added to the system. Chloramphenicol or *p*-fluorophenylalanine added to the cells while they were oxidizing octadecane caused a progressive fall off in rate to that of the endogenous respiration. From the above data it was deduced that at least one of the enzymes involved in the breakdown of paraffin hydrocarbons by the pseudomonad is unstable, breaking down with a half-life of about 30 min, but is normally resynthesised by the cell. In absence of NH_4^+, or in presence of chloramphenicol or *p*-fluorophenylalanine the enzyme cannot be synthesized and the overall rate of paraffin oxidation falls. These findings may be compared with those of PROCTOR[1], who found essentially similar results with a hexadecane-utilizing organism, but was able to detect a reduced rate of oxidation of paraffin hydrocarbon, corresponding to conversion to the corresponding primary alcohol, in the chloramphenicol-inhibited system.

Cells harvested by centrifugation following growth in octadecane–ammonia–salts medium were found to contain about 30% lipid, 90% or more of which was non-saponifiable. The fatty acids obtained were methylated, and the methyl esters examined by gas–liquid chromatography. The non-saponifiable material was also analysed by this technique. Comparison of the results with those obtained from cells grown on a glucose–broth–yeast extract medium, and containing only 3% total cell lipid, showed two major differences in the fatty acid methyl ester fractions. The paraffin-grown cells contained stearic acid, present only in traces in the control cells, and a larger amount of palmitic acid. The control cells contained virtually no volatile non-saponifiable material but paraffin-grown cells contained about 26–30% non-saponifiable material which was shown to contain from 1 to 50% octadecanol. Octadecanol was recrystallized from petroleum ether and the melting point of 58.5° was not depressed by addition of an authentic sample of octadecanol. Chromatographically the isolated material was indistinguishable from octadecanol on both polyvinyl acetate and Apiezon-L liquid phases. The conditions under which octadecanol is produced are still under investigation, but it would appear that moribund cells contain the largest amounts, thus supporting the theory that continuing protein synthesis is necessary for the further oxidation of octadecanol. The presence of traces of octadecanol in all cell preparations from cultures using octadecane as carbon source, and its total absence from broth-grown cells may be taken as evidence that this compound is an intermediate in the breakdown of octadecane by this pseudomonad.

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[1] M. H. PROCTOR, *Biochim. Biophys. Acta*, 42 (1960) 559.

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A paraffin-oxidizing pseudomonad

A pseudomonad enriched from a Dublin soil in a mineral salts–paraffin medium has been found rapidly to oxidize hexadecane. Other alkanes of longer or shorter chain length were not oxidized without lag by either paraffin- or hexadecane-grown cells. Quantitative manometric observations showed that hexadecane was incompletely oxidized, and that a definite "break" in the oxidation rate corresponded to the consumption of 0.51 mole O_2/mole of substrate, which in turn implied production of cetyl alcohol as a major metabolic intermediate. Titration of the supernatant medium from cultures in early growth on mineral salts–paraffin medium indicated negligible acid production, but a faint dichromate reaction suggested that an alcohol had accumulated. In older cultures esters were detected but not identified.

Organisms in the late exponential growth phase in a hexadecane–mineral salts medium with little added nitrogen (20 μg/ml ammonium nitrogen) were harvested, and the ethanol–ether-soluble fraction extracted from the air-dried cells. This fraction which accounted for 20 % of the dry weight was resolved to saponifiable and non-saponifiable lipids, and from the latter cetyl alcohol was isolated by crystallization from ethanol as flat plates, m.p. 49° which was not depressed by mixture with authentic cetyl alcohol. On nitrogen-rich medium (100 μg/ml ammonium nitrogen) the cell yield was increased but total lipids were reduced to only 4 % of the dry weight, and no cetyl alcohol was recovered.

In contrast to the manometric behaviour of cells grown with ample nitrogen and hexadecane as carbon and energy source, cells grown on nitrogen-deficient medium with hexadecane as carbon source consumed a maximum of 0.5 mole oxygen/mole hexadecane; however, addition of ammonium nitrogen (100 μg) to these nitrogen-deficient cells in the respirometer allowed further oxidation of the hexadecane. This further oxidation was sensitive to chloramphenicol, dinitrophenol, and azide, suggesting that it involved synthesis of a protein, probably an enzyme acting on cetyl alcohol as substrate.

This system provides an example of the synthesis of a series of simultaneously induced enzymes[1] the formation of one of which is influenced not only by the accumulation of its substrate, but also by the nitrogen level of the cells[2].

Publication of these data, obtained in 1957 in the Department of Bacteriology, Trinity College, Dublin, has been unavoidably delayed and is now made since it provides independent confirmation, using different techniques, of the recent elegant studies of KALLIO and co-workers[3].

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[1] R. Y. STANIER, *J. Bacteriol.*, 54 (1947) 339.

[2] A. I. VIRTANEN, *Ann. Acad. Sci. Fennicae, A*, 36 (1949) 1.

[3] J. E. STEWART, R. E. KALLIO, D. P. STEVENSON, A. C. JONES, AND D. O. SCHLISSLER, *J. Bacteriol.*, 78 (1959) 441.

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The role of DPN in the enzymic phosphorylation of creatine

A soluble enzyme system from rat skeletal muscle can phosphorylate creatine with 1,3 diphosphoglycerate in the absence of added ADP[1]. Using PEP as phosphate donor, ADP is absolutely required, but DPN may replace it. Related results have been reported by others[2,3].

Analytical procedures, substrate and enzyme preparations have been described[1]. The enzyme fraction used was the protein which is precipitated between 35 and 65 % of ammonium sulfate saturation. It was dialysed and treated with charcoal[1].

Creatine phosphorylation by PEP requires a nucleotide. Our rat-muscle preparation contains enough cofactor for this reaction, if not previously treated with charcoal (Table I). Pure CrK[4] plus PyK (Boehringer and Soehne, Mannheim) cannot replace the rat-muscle preparation in the presence of DPN. Apyrase[6] treatment inactivates ADP, but does not affect DPN as a transphosphorylation agent.

TABLE I

PHOSPHOCREATINE SYNTHESIS BY PEP WITH CRUDE AND PURIFIED ENZYMES

0.025 *M* Tris-HCl buffer, pH 7.5; 0.05 *M* creatine; 0.01 *M* $MgCl_2$; 0.005 *M* KCl; 0.0035 *M* PEP; rat-muscle enzyme, 2.4 mg/ml; PyK, 0.1 μg/ml; CrK, 200 μg/ml; volume, 2 ml. After 20 min at 37° reaction was stopped with 5 % trichloroacetic acid and PCr was determined. Apyrase treatment: 0.001 *M* ADP or 0.01 *M* DPN were treated with 5 mg potato apyrase for 10 min at 37°, and deproteinized with 5 % trichloroacetic acid, which was removed with ether. The neutralized aqueous phase was used for PCr synthesis at the indicated concentration.

Enzyme used for PCr synthesis	*Addition*	*Concentration of addition* ($M \times 10^4$)	*PCr synthesis* *μmoles/ml/20 min*
Pyk + Crk	ADP	0.5	2.30
Pyk + Crk	DPN	5.0	0.0
Rat muscle	None	—	0.0
Rat muscle, charcoal treatment omitted	None	—	0.35
Rat muscle	ADP	0.5	1.55
Rat muscle	Apyrase-treated ADP	0.6	0.0
Rat muscle	DPN	5.0	1.60
Rat muscle	Apyrase-treated DPN	5.0	1.55

The concentration of DPN[6] does not change during the experiment. However, the experiments in Fig. 1 suggest that DPN is not directly involved in transphosphorylation. The rat-muscle enzyme was preincubated with ADP or DPN in a system identical to one described in Table I, but lacking PEP. After different periods of preincubation, PEP was added (0.004 *M*) and the complete system was now incubated for another 2 min before determining PCr (Fig. 1 A, B). In another set of experiments (Fig. 1 D, E), ADP or DPN were boiled in 1 *N* HCl for various lengths of time, then neutralized and used for PCr synthesis. It may be seen that ADP and DPN behave differently in both sets of experiments.

Different moieties of the DPN molecule were tested; only ADP-ribose[7] is effective in transphosphorylation (Fig 1 C).

Our data suggest the presence in rat skeletal muscle of a DPN-splitting enzyme.

Abbreviations: ADP, adenosine diphosphate; PEP, phospho(enol)pyruvate; CrK, creatine phosphokinase; Pyk, pyruvic kinase; DPN, diphosphopyridine nucleotide; PCr, phosphocreatine; Tris, Tris(hydroxymethyl)aminomethane.

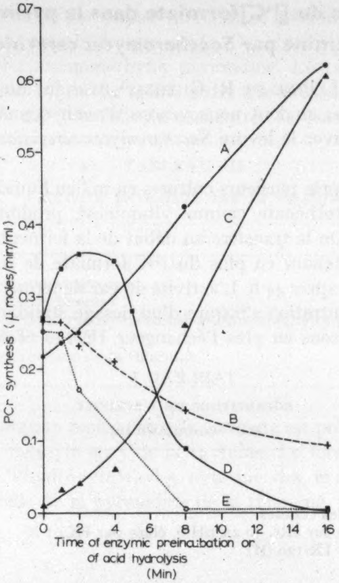

Fig. 1. Phosphocreatine synthesis after pretreatment of the nucleotides. Conditions as in Table I. Incubation time for PCr synthesis, 2 min. Treatment of nucleotides, see text. Nucleotide concentration: A, DPN ($7.5 \cdot 10^{-4}$ *M*), preincubated with muscle enzyme; B, ADP ($5.0 \cdot 10^{-5}$ *M*), preincubated with muscle enzyme; C, ADP-ribose ($1.6 \cdot 10^{-4}$ *M*), preincubated with muscle enzyme; D, HCL-treated DPN ($5 \cdot 10^{-4}$ *M*); E, HCL-treated ADP (10^{-5} *M*).

The split product may be not identical to ADP either in its structure or in the way it is bound by the enzymes involved in phosphate transfer.

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[1] O. CORI, A. TRAVERSO CORI, M. LAGARRIGUE AND F. MARCUS, *Biochem. J.*, 70 (1958) 683.
[2] P. OEHLEMYER AND S. OCHOA, *Biochem. Z.*, 293 (1937) 338.
[3] N. B. CHERNYAK, *Biokhimiya*, 19 (1954) 50.
[4] S. KUBY, L. NODA, AND H. A. LARDY, *J. Biol. Chem.*, 209 (1954) 191.
[5] P. S. KRISHNAN AND W. L. NELSON, *Arch. Biochem.*, 19 (1948) 65.
[6] N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Academic Press, New York, 1955, p. 660.
[7] S. ROSENBERG AND M. R. BOVARNICK, *J. Biol. Chem.*, 211 (1954) 763.

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Incorporation du [^{14}C]formiate dans la pyrimidine de la thiamine par *Saccharomyces cerevisiae*

Une publication de M. J. Pine et R. Guthrie[1], traitant du même problème dans le cas de *Bacillus subtilis* et dont nous venons d'avoir connaissance, nous incite à rapporter nos résultats avec la levure *Saccharomyces cerevisiae* dans leur état actuel d'avancement.

Une levure haute, après plusieurs cultures en milieu liquide défini, ne contenant inositol, biotine et pantothénate comme vitamines[2], produit 1–2 μg de thiamine par g de poids humide. On la transfère au début de la fermentation vigoureuse dans un milieu identique contenant en plus du [^{14}C]formiate de sodium (83 mg/ml). On recueille un poids double après 44 h. L'activité du gaz de fermentation est négligeable. Notre méthode de concentration s'inspire d'un dosage standard[3], et de la technique de Vincent[4]. Nous utilisons en plus l'échangeur IR-120 (Tableau I), et terminons par une chromatographie sur papier[5]. Après dilution par de la thiamine inactive, on fait la coupure classique en thiazole (presque inactif) et acide aminopyrimidinesulfonique, qui est actif et conserve son activité par recristallisation et désamination hydrolytique. Le sel de calcium de l'acide désaminé, *dérivé neutre*, se comporte de même (Tableau II).

TABLEAU I

RÉPARTITION DE L'ACTIVITÉ

(10^{-3} comptes/min)

Total introduit	8000
Activité résiduelle du milieu	1600
Extrait acide H_2SO_4 0.1 *N* des cellules	2700
Fraction "thiamine" retenue sur IRC 50 au pH 6, éluée par HCl 1 *N*	25
Passage de cette fraction sur IR-120 (H)	
Elué par HCl 1 *N*	3.8
par HCl 1.2 *N*	20
par HCl 3 *N*, (fraction thiamine), calculé par différence	1.2

TABLEAU II

ACTIVITÉ ENTRAÎNÉE PAR LA THIAMINE

	Comptes/min/mg
1-Picrate du fragment thiazole	0.2
2-Acide méthyl-2-amino-4-pyrimidine méthylsulfonique-5, 1ère, 2ème, et 3ème recristallisations	25, 20, 21
3-Acide méthyl-2-hydroxy-4-pyrimidine méthylsulfonique-5	19
4-Sel de calcium, 1ère et 2ème recristallisations (autre compteur)	61, 63
Poids de diluant: 10 mg	
Activité liée à la pyrimidine: 117	

Nous n'avons pas voulu fonder nos conclusions sur un seul groupe de dérives, car la levure fabrique très peu de thiamine, dont l'activité ne peut être qu'une faible fraction de celle du fond contaminant. Dans une autre expérience, nous purifions la thiamine par électrophorèse sur papier (pH 3.9) et nous dégradons la thiamine par oxydation en disulfure, puis hydrolyse acide. On obtient ainsi le amino-4 bichlorhydrate de la méthyl-2 aminométhyl-5 pyrimidine. Le taux d'incorporation, non modifié par recristallisation, est du même ordre que précédemment. L'activité de la pyrimidine représentait 24 % de celle du concentré (Tableau III).

TABLEAU III

ACTIVITÉ ENTRAÎNÉE PAR LA THIAMINE

	Comptes/min
Après concentration par chromatographie	2140
Après concentration par électrophorèse	730
Activité du bichlorhydrate de la méthyl-2-amino-4-aminométhyl-5-pyrimidine, par mg, (poids de diluant, 25 mg)	11.3
Activité totale liée à la pyrimidine de la thiamine	173

Le taux d'incorporation moléculaire du formiate est près de 20 %. Or le formiate est un précurseur du méthyle en 5 de la thymine. Le formol est un précurseur de l'hydroxyméthyle de l'hydroxyméthyl-5 cytosine des bactériophages[6], qui a une structure voisine de celle de la pyrimidine de la thiamine. Si l'essentiel de l'activité que nous avons trouvée est portée par le méthylène en 5 de nos dérivés, il est possible que la pyrimidine de la thiamine se fasse comme les autres pyrimidines, et que l'hydroxyméthyl-5 cytosine soit un intermédiaire. Pour la méthylation en C_2, il faudrait alors un donneur nucléophile. Une condensation avec l'acétylcoenzyme A donnerait l'acide (I) qui devrait être facilement décarboxylable.

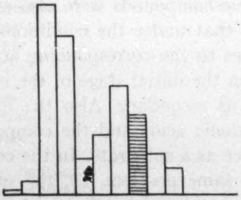
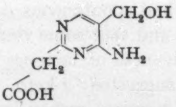


Fig. 1. Chromatogramme du concentré de thiamine. Activités relatives. La thiamine est dans la bande hachurée.



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[1] M. J. Pine et R. Guthrie, *J. Bacteriol.*, 78 (1959) 545.
[2] R. S. W. Thorne, *J. Inst. Brewing*, 55 (1949) 18.
[3] Analytical Methods Committee, *Analyst*, 76 (1951) 127.
[4] J. E. Vincent, *Rec. Trav. Chim.*, 76 (1957) 780.
[5] D. et N. Siliprandi, *Biochim. Biophys. Acta*, 14 (1954) 52.
[6] J. G. Flaks et S. S. Cohen, *Biochim. Biophys. Acta*, 25 (1957) 667.

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Substrate specificity of DPN-L-gulonic acid dehydrogenase

The enzymic conversion of L-gulonic acid to L-xylulose in mammalian tissues has been investigated by several workers[1-3]. ISHIKAWA in our laboratory first purified the enzyme, DPN-L-gulonic acid dehydrogenase, from guinea-pig liver and studied its properties[2]. ASHWELL *et al.* also reported the purification and properties of the enzyme from hog kidney[3]. The substrate specificity of the enzyme, however, has not yet been investigated satisfactorily by these authors. In the course of recent studies in our laboratory concerning a series of aldonic acids, elucidation of the substrate specificity of the enzymes participating in the metabolism of the aldonic acids was required. The present paper deals with this problem in respect to DPN-L-gulonic acid dehydrogenase.

The enzyme was purified from guinea-pig liver exactly according to the purification procedures already described by ISHIKAWA[2]. The assay method was also the same as his description. Mainly aldonic acids: trionic, tetronic, pentonic, hexonic and heptonic acids, and some related compounds were examined as substrates. The results are shown in Table I. Of these compounds tested L-gulonate, D-gluconate, D-mannonate, L-idonate, D-xylonate, D-lyxonate, DL-erythronate, L-threonate and DL-β-hydroxybutyrate were effective. In all these compounds the hydroxyl group of the C-3 has a L-configuration and the hydrogen of the C-4 occupies a L-position. Those in which both hydroxyl groups of the C-3 and C-4 have L-configurations were not effective. Lactones corresponding to the above compounds were also effective, although to a much less extent, but considering that under the conditions of our assay method spontaneous degradation of lactones to the corresponding acids was inevitable and that some time lag was observed in the initial stage of the reaction, the effectiveness of lactones might be regarded as secondary. Also the following might be suggested: 2-keto, as well as 5-keto aldonic acids and the compound in which the terminal carbon is aldehyde do not serve as a substrate. In the course of our present study SMILEY *et al.* reported on the same problem, *i.e.* the substrate specificity of the enzyme and the reverse reaction catalyzed by the enzyme[4]. They concluded that the enzyme is active upon all hexonic, pentonic and tetronic acids examined in which the hydroxyl group of the C-3 has a L-configuration, and that the reverse reactions from 2,3-diketo-L-gulonate, 2,3-diketo-D-gluconate, 2,3-diketo-D-glucoheptonate and acetoacetate to the corresponding C-3 hydroxy acids occur. In our study, however, the enzyme is not active upon the compounds such as D-galactonate, D-talonate and L-arabonate in which the hydroxyl group of the C-3 has a L-configuration. Therefore, we are of opinion that the simple condition of the C-3 L-hydroxyl group is not sufficient, but the configuration of C-3 L-hydroxy and C-4 L-hydrogen are necessary for the substrate. We also tried to examine spectrophotometrically the reverse reaction catalyzed by the enzyme using 2,3-diketo-L-gulonate, 2,3-diketo-D-gluconate and acetoacetate as a substrate. However, under various conditions examined the reverse reaction was not found. The difference between the observations of SMILEY *et al.* and ours might be attributed to the difference of the source of the enzyme.

D-Gluconate was previously reported ineffective, but it has now been found that

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Abbreviations: DPN, diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

TABLE I

SUBSTRATE SPECIFICITY OF DPN-L-GULONIC ACID DEHYDROGENASE

The reaction mixture contained Tris buffer, 100 µmoles; $MnCl_2$, 0.2 µmole; DPN, 0.65 µmole; substrate, 10 µmoles, or 20 µmoles when the substrate was DL-form; enzyme, 0.1 ml (approx. 100 units*). Total vol., 3.0 ml, pH 8.4. Incubation for 5 min at 25°. The change in absorbancy at 340 mµ due to the reduction of DPN was determined.

Substrate	*Relative activity of that of L-gulonate as 100*	*Substrate*	*Relative activity of that of L-gulonate as 100*
DL-Glycerate	0		
DL-Erythronate	17.1		
		L-Threonate	7.8
DL-β-Hydroxybutyrate	14.4		
D-Ribonate	0		
D-Arabonate	0	L-Arabonate	0
D-Xylonate	15.5	L-Xylonate	0
D-Lyxonate	44.6	L-Lyxonate	0
D-Allonate	0		
D-Altronate	0		
D-Gluconate	19.0	L-Gluconate	0
2-Keto-D-gluconate	0		
5-Keto-D-gluconate	0		
D-Glucuronate	0		
D-Mannonate	5.1	L-Mannonate	0
D-Gulonate	0	L-Gulonate	100
		2-Keto-L-gulonate	0
		Methyl 2-keto-L-gulonate	0
D-Idonate	0	L-Idonate	68.8
D-Galactonate	0	L-Galactonate	0
D-Talonate	0		
D-α-Glucoheptonate	0		
D-β-Glucoheptonate	0		

* Unit is as defined by ISHIKAWA[2].

this compound was 20 % as active as L-gulonate. The reaction product from D-gluconate is probably D-ribulose. This reaction is very interesting in connection with the metabolism of non-phosphorylated sugars. The identification of the reaction product is now under investigation in our laboratory.

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[1] S. ISHIKAWA AND K. NOGUCHI, *J. Biochem.*, 44 (1957) 465.
[2] S. ISHIKAWA, *J. Biochem.*, 46 (1959) 347.
[3] G. ASHWELL, J. KANFER AND J. J. BURNS, *J. Biol. Chem.*, 234 (1959) 472.
[4] J. D. SMILEY AND G. ASHWELL, *Federation Proc.*, 19 (1960) 85.

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Chondroitin sulfate B metabolism

Relatively little information is available regarding the detailed mechanisms involved in the biosynthesis of the connective-tissue mucopolysaccharides. Studies performed in several laboratories have implicated uridine nucleotides as intermediates in these transformations[1,2]. Although available evidence indicates that the acid polysaccharides in skin (chondroitin sulfate B and hyaluronic acid) are in a dynamic metabolic state[3], the mechanism of breakdown of these polymers is not understood. The present preliminary report describes the behavior of chondroitin sulfate B in the presence of extracts of rabbit- or chick-embryo skin.

Chondroitin sulfate B labeled with ^{35}S in the sulfate group was prepared from rabbit skin obtained from animals injected with 500 μC of $^{35}SO_4^=$ 48 h prior to sacrifice. The polysaccharide was purified by conventional methods and exhibited satisfactory analyses[4]. Extracts were prepared from 2-week-old rabbit skin, or 21-day-old chick-embryo skin by homogenizing in a Waring Blendor for 2 min with 5 vol. 0.15 M KCl containing $5 \cdot 10^{-4}$ M glutathione. The extracts were centrifuged for 15 min at 18,000 × g and the supernatant solution treated with 0.1 vol. 2 % protamine sulfate. After standing for 15 min in an ice bath, the precipitate was removed by centrifugation and discarded. The protamine supernatant, 3–5 mg protein/ml, was used for the experiments described.

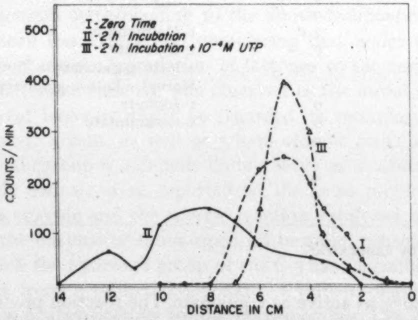

Fig. 1. Paper electrophoresis of chondroitin sulfate B incubations. Paper-electrophoretic patterns of incubation mixture containing [^{35}S]CSB, 1.0 μmole, 70,000 counts/min/mg; phosphate buffer (pH 7.5), 50 μmoles; EDTA, 0.5 μmole; enzyme protein, 0.4 mg. After incubation for 2 h, the protein was removed by shaking with $CHCl_3$–butanol (9:1) and centrifugation. The polysaccharide material was precipitated from the supernatant by the addition of 2 vol. 95 % ethanol containing 0.5 N acetic acid. The precipitate was harvested by centrifugation, dissolved in 1.0 M K_2CO_3 and the resulting solution subjected to paper electrophoresis at 500 V for 45 min in 0.1 M acetate buffer, pH 5.0. When migration was complete, the strips were dried, sectioned and assayed for radioactivity in a Tri-Carb liquid scintillation counter (Packard Instrument Co., La Grange, Ill.) with toluene as solvent and PPO:POPOP (40:1) as scintillators. This method is desirable because of high efficiency (40 %) and elimination of self-absorption corrections. Since the compounds studied are not soluble in toluene, subsequent chemical assay for uronic acid content of eluted material was also performed and showed patterns coincident with radioactivity.

Abbreviations: ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; CTP, cytosine 5'-triphosphate; UTP, uridine 5'-triphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene; CSB, chondroitin sulfate B; EDTA, ethylenediaminetetraacetic acid.

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Incubation of choindroitin sulfate B with rabbit-skin enzyme followed by paper electrophoresis led to the pattern illustrated in Fig. 1. Degradation of the polysaccharide to a mixture of lower-molecular-weight fragments is indicated by the appearance of variable amounts of more rapidly migrating components. In contrast, when the incubation was performed in the presence of UTP, the polysaccharide fraction appeared to be of high molecular weight and less polydisperse than the starting material.

The UTP effect is highly reproducible and the nucleotide cannot be replaced by ATP or GTP in comparable concentration. UTP also appears to stimulate sulfate incorporation in a nucleotide-specific manner[5]. The effect of UTP on counts recoverable by ethanol precipitation is indicated in Table I.

Although the paper-electrophoretic patterns indicated the sulfate group to be still bound to an oligosaccharide unit, extracts were examined for chondrosulfatase activity (Table II). None were detected.

TABLE I

RECOVERY OF CHONDROITIN SULFATE B BY ETHANOL PRECIPITATION

Conditions as described in Fig. 1. After completion of incubation and removal of protein, sufficient ethanol–acetic acid was added to precipitate 50% of the initial polysaccharide. The resulting precipitate was harvested and assayed for radioactivity in a gas flow counter.

	Zero time	*2-h incubation*	*2 h + 10^{-4} M UTP*
Crude extract	1847	1284	1787
Protamine supernatant	1855	826	1696

TABLE II

CHONDROSULFATASE ACTIVITY OF SKIN EXTRACTS

Incubation mixture contained imidazole (pH 7.0), 100 μmoles; EDTA, 2 μmoles; [^{32}S]CSB, 4 μmoles; [^{35}S]CSB, 2.6·10^{6} counts/min protein, 1.3, 2.6 mg. At time *t*, 100 μmoles K_2SO_4 and excess $BaCl_2$ were added. The $BaSO_4$ was extensively washed and counted.

	I	*II*
Zero time	152	319
2-h incubation	146	327

In general, activities of chick-embryo-skin extract and rabbit-skin extract were similar. Comparable transformations were not observed with chondroitin sulfate A (cartilage) as substrate for the skin extracts. The nature of the intermediate products is under investigation.

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[1] L. GLASER AND D. H. BROWN, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 5.
[2] A. MARKOVITZ, J. A. CIFONELLI AND A. DORFMAN, *J. Biol. Chem.*, 234 (1959) 2343.
[3] S. SCHILLER, M. B. MATHEWS, J. A. CIFONELLI AND A. DORFMAN, *J. Biol. Chem.*, 218 (1956) 139.
[4] K. MEYER, E. A. DAVIDSON, A. LINKER AND P. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506.
[5] E. A. DAVIDSON AND J. G. RILEY, *J. Biol. Chem.*, in the press.

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